Investigating the role of the human glomerular endothelial cells in juvenile-onset lupus nephritis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy.

By

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I wish to dedicate this thesis to my partner and to my family.

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I. ABSTRACT

Introduction: Lupus nephritis (LN) is one of the most serious and significant chronic complications of juvenile-onset systemic lupus erythematosus (JSLE) affecting up to 80% of patients. The renal glomerulus is severely affected in LN leading to chronic inflammation and glomerulonephritis (GN). GN can affect all parts of glomeruli, including the glomerular endothelial cells (GEnCs), which together with podocytes and their shared glomerular basement membrane (GBM), form the glomerular filtration barrier (GFB).

Aims: It was hypothesised that human GEnCs are not just passive bystanders but are actively involved in the perpetuation of renal inflammation in LN. Thus, the main aim of this project was to elucidate the pro-inflammatory involvement of human GEnCs in LN, utilising *in vitro* models of conditionally immortalised human GEnCs (ciGEnCs).

Materials and Methods: The ciGEnCs were treated with cytokines known to be involved in LN pathogenesis (TNF-α, IL-1β, IFN-α, IFN-γ, IL-6, IL-13, VEGF) and LPS. Subsequently, ciGEnC expression, secretion and production of various pro-inflammatory proteins were tested via real-time PCR, ELISA and flow cytometry. Activation of the pathways involved were investigated via Western blotting and immunofluorescence. To assess the role of the renal microenvironment, ciGEnCs were treated with JSLE (active/inactive LN) and paediatric healthy control sera and with conditioned media from cytokine-/LPS-stimulated mesangial cells, podocytes, human neutrophils, macrophages and THP-1 cells; mRNA expression and protein secretion of pro-fibrotic and pro-inflammatory genes/proteins was tested. The effect of the novel urinary LN biomarkers (AGP, CP, TF, L-PGDS, MCP-1, NGAL) on ciGEnC pro-inflammatory gene expression and ciGEnC viability was tested, following treatments with each biomarker alone, or all in combination.

Results: The ciGEnCs were found to significantly upregulate ($p \leq 0.05$) the production of most pro-inflammatory proteins tested including cytokines, chemokines and cell adhesion molecules (CAMs) following cytokine stimulation confirming a role for GEnCs in perpetuating the inflammatory response. The cytokines found to have a robust effect on ciGEnC activation ($p \leq 0.05$) were TNF-α, IL-1β, and IFN-γ (IL-13 was only able to significantly induce surface VCAM-1 and sVCAM-1 production), therefore these three cytokines were further assessed in terms of pro-inflammatory transcription factor activation. TNF-α and IL-1β induce their effects through NF-κB activation; while IFN-γ acts via STAT-1 activation. The JSLE sera exerted a moderate to low effect compared to cytokines but were able to upregulate the expression of certain pro-fibrotic and pro-inflammatory genes. THP-1 cell conditioned media induced significant changes in ciGEnC pro-inflammatory proteins secretion suggesting macrophage involvement in the pathogenesis of LN. The novel urinary biomarkers were not found to affect ciGEnC pro-inflammatory activation and ciGEnC viability.

Conclusion: This study identified the human GEnCs as important pro-inflammatory players in LN-associated renal disease. The GEnCs can actively respond to pro-inflammatory stimuli and are involved in immune cell activation and recruitment through cytokine and chemokine secretion and upregulation of adhesion molecules.

II. ACKNOWLEDGEMENTS

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most important pillars of a successful professional collaboration are mutual respect and tolerance and I think we were able to achieve both.

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Every person has their own personal story and their own roots; mine are back in Greece, in the small seaside town of Chalkida where I was born and raised by two most wonderful parents, my dad Vlasis and my mum Vasileia. They were my first and biggest supporters who taught me to read my favourite fairytales since the age of five, whose endless love shaped my character and whole personality and who boosted me and gave me strength in every step along the way. Without them, few things would have been the same for me today and they have always been my role-models in life.

Along with them, my younger brother Christos and all my good friends have been my loyal fans since school and every year, their admiration grows even stronger. It feels me with joy when they naïvely say to me: "Looking forward to the day you're gonna get the Nobel Prize"! It is so nice to be loved and cherished and to feel special even if you are just another young scientist among many others who are struggling to do their best in this world!

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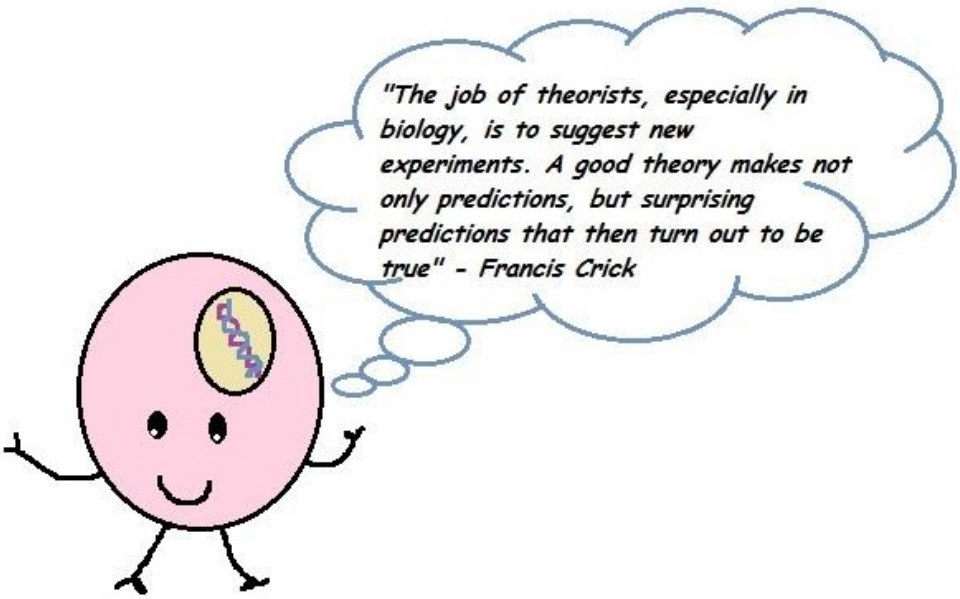
yes, be ready for a very cliché phrase- feels like I've been knowing him for many more years. Even at times that almost everyone would doubt me, including myself, Yiannis would never lose his faith in me. He is my real-life coach and thanks to him and to our endless conversations about life, about science, experiments, techniques and thanks to his amazing suggestions, I have been able to spectacularly improve in so many aspects of my life! Thank you, Yiannis, for being my biggest fan ever; I am your biggest fan, too. Together, I am sure we're going to have the time of our lives!

Finally, cheers to every single person in this world who's been struggling every day to make their dreams come true.



Vivian Dimou, June 2019

III. DECLARATION

This thesis is a result of my own work performed during the course of my studies in the Department of Women's and Children's Health, Institute of Translational Medicine, University of Liverpool, between September 2015 and August 2018. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or qualification. All work described was performed by me except where clearly indicated. The thesis was written wholly by me under guidance of my supervisors Prof Michael W Beresford, Dr Rachael D Wright, Dr Angela Midgley and Prof Matthew Peak.

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VI. ABBREVIATIONS

A488 - Alexa Fluor 488

A568 – Alexa Fluor 568

Ab -Antibody

ACTB – beta-actin

AGP - Alpha-1-acid glycoprotein

AKI - Acute Kidney Injury

ANCA – Anti-neutrophil cytoplasmic antibodies

Ang-1 - Angiopoietin-1

anti-dsDNA - anti-double stranded DNA

Anx V - Annexin V

AP-1 - Activator protein-1

APC - Allophycocyanin

ASLE – Adult-onset systemic lupus erythematosus

BCA - Bicinchoninic acid

BILAG - British Isles Lupus Assessment Group

bp - base pairs

BSA - Bovine Serum Albumin

CCL2 - CC-chemokine ligand 2

CCL3 - CC-chemokine ligand 3

CD31 - Cluster of differentiation 31

CD99 - Cluster of differentiation 99

cDNA – Complementary DNA

ci – conditionally immortalised

ciGEnCs – conditionally immortalised Glomerular Endothelial Cells

CKD – Chronic Kidney Disease

CNS – Central Nervous System

COL1A1 - Collagen type I alpha 1 chain

COL1A2 - Collagen, type I, alpha 2

COL3A1 - Collagen, type III, alpha 1

COL4A1 - Collagen, type IV, alpha 1

COX-2 - Cyclooxygenase-2

CP - Caeruloplasmin

CPRD - Clinical Practice Research Datalink

CSF-1 – Colony Stimulating Factor-1

CSF-2 - Colony Stimulating Factor-2

Ct – Cycle threshold

CXCL10 - C-X-C motif chemokine 10

DAPI - 4',6-diamidino-2-phenylindole

DMSO - Dimethyl Sulfoxide

dNTP – deoxyribose nucleoside triphosphates

dsDNA – double-stranded DNA

DTT - Dithiothreitol

ECL - Enhanced chemiluminescence

ECM - Extracellular Matrix

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

ESL - Endothelial Surface Layer

FBS – Fetal Bovine Serum

FC – Flow Cytometry

Fc - Fragment crystallisable

FcR - Fc receptor

FITC - Fluorescein Isothiocyanate

FLS - Fibroblast-like synoviocytes

FN - Fibronectin

FSC – Forward Scatter

GBM - Glomerular Basement Membrane

GEnCs – Glomerular endothelial cells

GFB – Glomerular Filtration Barrier

GFR – Glomerular Filtration Rate

GM-CSF - Granulocyte Macrophage-Colony Stimulating Factor

GN - Glomerulonephritis

GPCRs - G-protein-coupled receptors

h - hour(s)

H_2O_2 - Hydrogen Peroxide

HBMECs - Human Brain Microvascular Endothelial Cells

HBSS - Hank's Balanced Salt Solution

HCAECs – Human Coronary Artery Endothelial Cells

HCQ - Hydroxychloroquine

HCs - Healthy Controls

h-EGF – human Endothelial Growth Factor

h-FGF – human Fibroblast Growth Factor

h-IGF – human Insulin Growth Factor

HK – housekeeping

HLA - Human Leukocyte Antigen

HMECs - Human Microvascular Endothelial Cells

HOX - Homeobox

HRP – Horseradish Peroxidase

HSVECs – Human Sapenous Vein Endothelial Cells

hTERT - human telomerase reverse-transcriptase

HUAECs – Human Umbilical Artery Endothelial Cells

HUVECs - Human Umbilical Vein Endothelial Cells

ICAM-1 - Intercellular Adhesion Molecule-1

ICOS-L – Inducible Costimulator-Ligand

IF - Immunofluorescence

IFN – Interferon

IFN-α – Interferon-alpha

IFN-β - Interferon-beta

IFN-γ - Interferon-gamma

IL-1 – Interleukin-1

IL-10 – Interleukin-10

IL-12 – Interleukin-12

IL-13 – Interleukin-13

IL-17 - Interleukin-17

IL-18 - Interleukin-18

IL-1Ra – Interleukin-1 receptor antagonist

IL-1RI – Interleukin-1 receptor I

IL-1RII - Interleukin-1 receptor II

IL-1β - Interleukin-1 beta

IL-4 - Interleukin-4

IL-6 - Interleukin-6

IL-8 - Interleukin-8

IP-10 - Interferon-γ-inducible Protein 10

ITS - Insulin Transferrin Selenium

Iκ-Bα - Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha

JIA – Juvenile-onset Idiopathic Arthritis

JSLE – Juvenile-onset Systemic Lupus Erythematosus

LAMB1 - Laminin subunit beta 1

LAMB2 - Laminin subunit beta 1

LCN2 - Lipocalin-2

LEDs - Light Emitting Diodes

LN – Lupus Nephritis

L-PGDS - Lipocalin-type Prostaglandin D Synthase

LPR2 - Low-Density Lipoprotein-Related Protein 2

LPS - Lipopolysaccharide

MCP-1 – Monocyte Chemoattractant Protein-1

M-CSF - Macrophage-Colony Stimulating Factor

Mesangial cells – MCs

MHC - Major Histocompatibility Complex

MIP-1α - Macrophage Inflammatory Protein-1 alpha

MLCK - Myosin Light Chain Kinase

MMF – Mycophenolate Mofetil

MMP2 - Matrix Metalloproteinase 2

MMP7 - Matrix Metalloproteinase 7

MMP9 - Matrix Metalloproteinase 9

MPO - Myeloperoxidase

MPO-AAV – Myeloperoxidase - ANCA-associated vasculitis

mRNA – messenger RNA

MW- Molecular Weight

MΦ - Macrophages

NETs – Neutrophil Extracellular Traps

NF-κB - Nuclear factor-κB

NGAL - Neutrophil Gelatinase-Associated Lipocalin

NK cells – Natural Killer Cells

NO- Nitric Oxide

NOS-Nitric Oxide Synthase

ORM1 – Orosomucoid 1

PBMCs - Peripheral Blood Mononuclear Cells

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

pDCs – plasmacytoid Dendritic Cells

PDGF-B - Platelet-Derived Growth Factor Subunitt Beta

PDGFR-β - Platelet-Derived Growth Factor Subunit Beta receptor

PD-L1 - Programme Death-Ligand 1

PE - Phycoerythrin

PECAM-1 - Platelet-Endothelial Cell Adhesion Molecule-1

PECs - Parietal Epithelial Cells

PGD2 – Prostaglandin D2

PGH2 - Prostaglandin D2

PGI_2 - Prostaglandin I_2

Phospho-GSK-3β – Phoshpo Glycogen synthase kinase-3 beta

PI – Propidium Iodide

PMA - Phorbol 12-myristate 13-acetate

PPI - Protein-Protein Interaction

PS - Phosphatidylserine

PTGDS - Prostaglandin-H2 D-isomerase

PVDF - Polyvinylidene Difluoride

RA – Rheumatoid Arthrits

RPMI - Roswell Park Memorial Institute

RT – Reverse Transcription

RTase – Reverse Transcriptase

SDF-1 - Stromal-Cell Derived Factor-1

SEM - Standard Error Mean

SLE – Systemic Lupus Erythematosus

SSC – Side Scatter

STAT-1 - Signal transducer and activator of transcription-1

STAT-2 - Signal transducer and activator of transcription-2

SV40LT - Simian virus 40 large T-antigen

sVCAM-1 – soluble Vascular Cell Adhesion Molecule-1

TBP - TATA-Binding Protein

TBS - Tris-Buffered Saline

TBS-T - Tris-Buffered Saline-Tween-20

TF - Transferrin

TGF-β - Transforming Growth Factor-beta

TGF-β1 - Transforming Growth Factor-beta 1

Th1 - T-helper 1

Th17 - T-helper 17

Th2 - T-helper 2

TIMP1 - Tissue Inhibitor of Metalloproteinases 1

TLOs - Tertiary Lymphoid Organs

TLRs – Toll-like receptors

Tm – melting temperature

TMB - Tetramethylbenzidine

TNFR1 – Tumour Necrosis Factor Receptor 1

TNFR1 – Tumour Necrosis Factor Receptor 2

TNF-α – Tumour Necrosis Factor-alpha

Tohoku Hospital Pediatrics-1 – THP-1

TUBB - Beta-Tubulin

UCLH - University College London Hospitals

VCAM-1 - Vascular Cell Adhesion Molecule-1

VEFGR2 – Vascular Endothelial Growth Factor Receptor 2

VEGF - Vascular Endothelial Growth Factor

VEGFA - Vascular Endothelial Growth Factor A

VEGR1 - Vascular Endothelial Growth Factor Receptor 1

VLA-4 - Very Late Antigen-4

vWF - von Willebrand factor

WB – Western blotting

WPBs - Weibel-Palade bodies

YWHAZ - 14-3-3 protein zeta/delta

β-actin - Beta-actin

ΔCt – Delta Ct

ΔΔCt – Delta Delta Ct

1. Chapter 1: Introduction

1.1. Renal physiology and function.

The kidneys are two bean-shaped organs, approximately the size of a human fist, located in the upper abdominal area, with a fundamental role in the maintenance of pH and whole-body fluid homeostasis[1]. They are also essential for the control of blood pressure, osmotic regulation, maintenance of electrolyte and acid-base balance, bone mineralisation, vitamin D synthesis, production of hormones such as calcitriol and erythropoietin, and enzymes such as renin[1].

In adults, each kidney is approximately 10.9 to 11.2 cm long[2] -the size of a human fist- with the left kidney being larger that the right kidney. In an observational study of 437 children aged between 0 and <13 years, the size of each kidney was assessed via sonography and each kidney was found to be approximately 5.3 to 8.5 cm long (with the left kidney again being larger than the right kidney)[3]. These findings also agree with other studies, worldwide[4]. In study of 947 children aged between 6 and 17 years, the size of each kidney was assessed via sonography and was found to be between 7.15 to 8.77 cm for the right kidney, and between 7.33 to 9 cm for the left kidney[5].

One of the most important homeostatic functions of the kidneys is blood filtration which leads to excretion of the body's metabolic waste products into the urine. Each adult human kidney is able to generate on average 1-2 liters of urine every day[1]. Compared to adults, children produce smaller volumes of urine per day and the amount of urine produced depends on age. In adults, the normal urine output (that is often used to assess renal function and it is age-dependent) is approximately 0.5 ml/kg/hour, whereas in newborns and infants up to 1 year it is 2 ml/kg/hour, in toddlers it is 1.5 ml/kg/hour and in older children and adolescents it is approximately 1 ml/kg/hour[6].

Urine filtration and concentration occur within the nephrons, the structural and functional renal units[1]. There are approximately $1x10^6$ nephrons in each kidney; each nephron consists of a glomerulus where initial blood plasma filtration takes place and a reabsorption tubular system (made up of the proximal convoluted tubule, the loop of Henle and the distal convoluted tubule) for urine concentration leading finally to a collecting duct which leads to the ureter[1] **(Figure 1.1**).

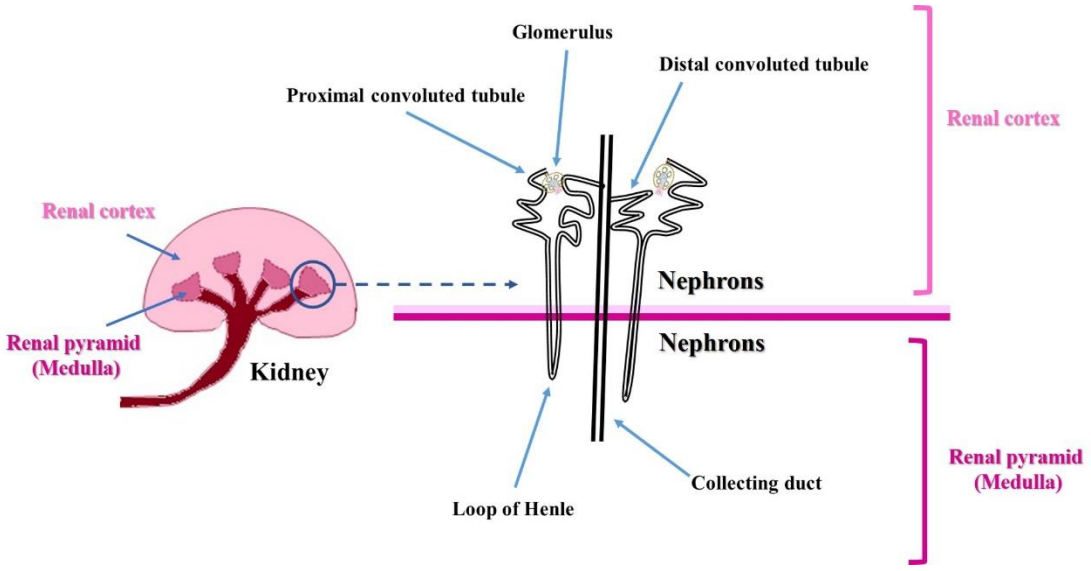


Figure 1.1 Nephron structure.

Each nephron, part of which is located in the renal pyramid (medulla) and part of which is located in the renal cortex, consists of a glomerulus (found in the renal cortex) and a series of connected tubules starting from the proximal convoluted tubule (attached to the glomerulus) and continuing to the U-shaped Loop of Henle and the distal convoluted tubule which empties the urine into the collecting duct.

The glomeruli are highly vascularised, tortuous tuft-like structures formed by parietal epithelial cells (PECs) consisting of a network of capillaries into which blood enters and exits via the afferent and efferent arterioles respectively. Each capillary is lined with glomerular endothelial cells (GEnCs) on its luminal side and with a specialised type of epithelial cells with interdigitating foot processes, the podocytes,

covering its exterior surface[7]. The GEnCs and podocytes contribute to a common extracellular matrix (ECM), known as the glomerular basement membrane (GBM)[7]. The mesangial cells (MCs) are found central to the capillaries forming a stalk-like structure, the mesangium. MCs contribute to the GBM by producing ECM, they provide structural support to the glomerular microvasculature and are responsible for the regulation of the blood flow through the capillaries (**Figure 1.2**).

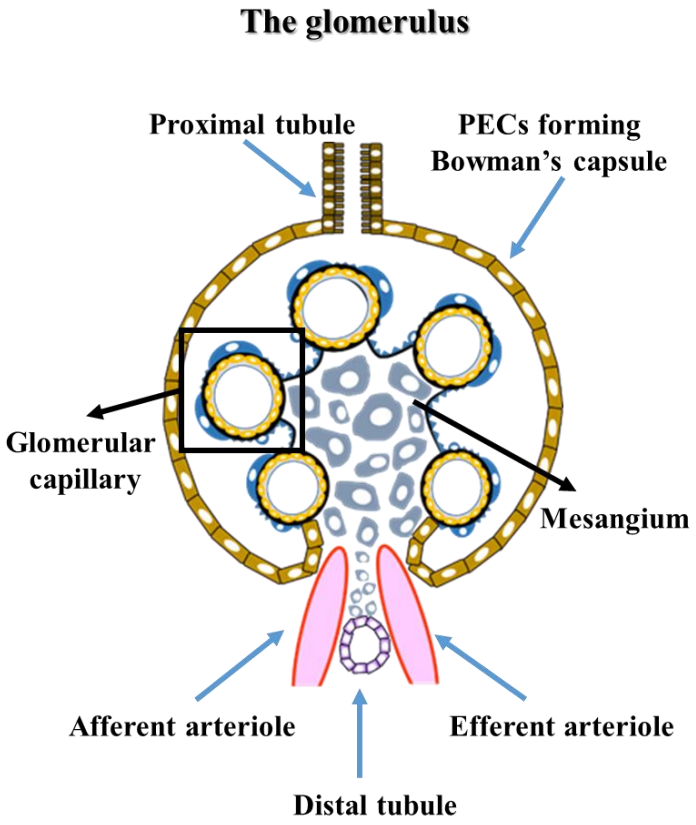


Figure 1.2 The glomerulus.

The glomerulus is a tuft-like structure formed by parietal epithelial cells (PECs), which are the constituents of the Bowman's capsule. Inside the Bowman's capsule are enclosed the glomerular capillaries. Blood enters and exits the glomeruli via an afferent and an efferent arteriole, respectively.

The GEnCs, the podocytes, and the GBM between them constitute the tri-layered glomerular filtration barrier (GFB). This is a biological semi-permeable sieve that

functions as a whole unit and selectively filters blood in order to generate the primary urinary filtrate. GEnCs are fenestrated and, together with the podocytes that interdigitate to form slit diaphragm junctions between neighboring foot processes, these form a physical barrier that permits only molecules of appropriate size/charge to cross the barrier[1]. The GBM and the GEnC glycocalyx (layer of glycoproteins surrounding the cells) form a highly negatively charged barrier that permits only positively charged molecules to pass through the sieve. Therefore, under physiological conditions the GFB permits only the passage of water and other small molecules (e.g. glucose, urea, amino acids and mineral ions) to the Bowman's capsule, whereas red blood cells (erythrocytes) and proteins (e.g. albumin) remain restricted inside the lumen and return to the circulation[8,9] (**Figure 1.3**).

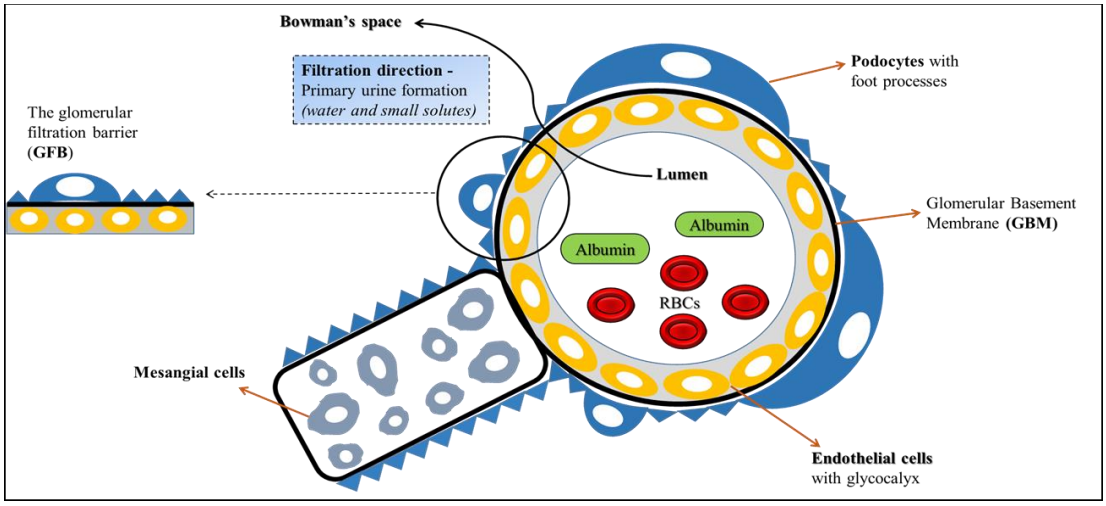


Figure 1.3 The renal glomerular capillary.

Each capillary consists of GEnCs lining the luminal side and podocytes covering its outer surface. These two cell types together with their common basement membrane form the GFB. The MCs provide structural support and regulate the blood flow.

The glomerular capillary tuft is enclosed by PECs forming the Bowman's capsule. The blood enters the glomerulus via an afferent arteriole, and it gets filtered through

the GFB. Subsequently, the proteins and erythrocytes return to the circulation through an efferent arteriole **(Figure 1.2)**. At the same time, the primary urinary filtrate composed mainly of water and small solutes, collects inside the Bowman's capsule and passes through the continuous renal epithelial tubular compartment starting from the proximal tubules, the U-shaped loop of Henle and the distal tubules to finally reach the collecting duct[1] **(Figure 1.1)**.

The majority of substances necessary for the normal function of the human body (e.g. water, electrolytes, amino acids and glucose) which are initially filtered through the glomerulus are reabsorbed by the renal tubules into the interstitial space and eventually back into the blood[1]. Waste products such as hydrogen, creatinine, ions and some drugs are secreted into the collecting duct where they form the urine which is excreted out of the body through the ureter. Both the reabsorption and excretion processes occur through a number of ion and water channels as well as transporters expressed by the renal tubules and collecting ducts, therefore both processes can either be active or passive[1].

1.1.1. GEnCs and their contribution to the GFB.

The GEnCs are characterised by the presence of transcellular pores or fenestrations of 60–80 nm in diameter occupying 30–40% of the cell surface, which allow for efficient and rapid filtration of large amounts of water across the endothelium [10,11]. The fenestrations are not just empty holes but are covered with a polyanionic gel-like fibrous mesh consisting of glycoproteins, proteoglycans (which consist of core proteins covalently bound to negatively charged heparan or chondroitin sulphate) and sialic acids anchored on the luminal side of the GEnC membrane[12,13]. Furthermore, blood plasma components such as hyaluronan, albumin, lumican and α-1-acid glycoprotein

(or orosomucoid 1; ORM1) are adsorbed into the glycocalyx[13]. The glycocalyx layer in combination with the adsorbed plasma components make a thicker filamentous coat (200nm) with strong negative charge which lines the luminal side of the glomerular endothelial microvasculature, known as the endothelial surface layer (ESL)[8,13]. The ESL is responsible for formation of the negative charge of the GFB that is important for limiting the passage of negatively charged solutes into the urine.

As the GEnCs are found in the blood plasma-renal tissue interface, they are the first GFB component to block passage towards the Bowman's capsule[1]. The ability of the glomerular endothelium to provide a first-line barrier inside the glomerulus was first demonstrated in 1976 by immunolabelling of albumin to track its location within glomeruli under physiological conditions[14]. This showed that during normal filtration and regular blood flow, albumin does not pass freely through the fenestrated glomerular endothelium but remains restricted inside the lumen. Furthermore, albumin density notably decreased at the endothelium compared to the capillary lumen, a finding that provided evidence for the pivotal role of GEnCs in albumin sequestration.

In vitro studies using conditionally immortalised human GEnCs (ciGEnCs) which have a 200nm glycocalyx,[15] as well as *ex vivo* and *in vivo* studies using cooled isolated perfused murine kidneys[16] and mice[17,18] respectively, have since provided evidence for the important role of ESL in albumin handling. Enzymatic removal of certain glycocalyx and ESL constituents such as heparan sulphate removal by heparinase in ciGEnCs[15] led to increased albumin permeation through the ciGEnC monolayers[19]. In addition, heparan sulphate removal by heparinase or hyaluronan removal by hyaluronidase in murine kidneys[16–18,20] led to higher albumin excretion *ex vivo* due to significant reduction of ESL anionic sites and thickness[16,20]. Moreover, this led to

higher urine protein excretion levels and albumin translocation to the podocyte space in *vivo* due to loss of glycocalyx volume [17,18]. Furthermore, in patients with type 1 diabetes mellitus, a decrease in the size of systemic endothelial glycocalyx volume has been correlated with increased levels of urinary protein excretion[21].

These data together suggest a pivotal ESL role in GFB albumin handling and sequestration inside the lumen and highlights the importance of normal glomerular endothelium function in the prevention of proteinuria.

1.1.2. Glomerular cell crosstalk

The formation and establishment of a fully functional GFB largely depends on the proper development of resident renal cells, as well as on the maintenance of the physiological crosstalk among them.

During the development of the glomerular vasculature, growing podocytes secrete Vascular Endothelial Growth Factor (VEGF) A (VEGFA). VEGFA has trophic and chemoattractant properties that promote the migration of endothelial precursor cells (angioblasts) which express on their surface the VEGFA cognate receptors, namely VEGFR1, VEGFR2 and neuropilin-1[22] to the area to begin angiogenesis. Furthermore, the podocytes and MCs secrete angiopoietin-1 (Ang-1) whereas the GEnCs express its respective receptor, Tie2[23,24]. Ang-1 is an endogenous endothelial growth factor that promotes glycocalyx synthesis as well as ESL thickening in the systemic endothelium, enhancing its barrier properties and leading to reduced albumin permeability[24,25] .

In addition, the GEnCs produce platelet-derived growth factor subunit B (PDGF-B) and MCs express its receptor PDGFR-β[26–28]. The interaction of PDGF-B with PDGFR-β is necessary for MC proliferation and the development of the glomerulus[29].

Finally, the development of an *in vitro* conditionally immortalised GEnC-podocyte co-culture model has provided evidence that the GEnCs have the ability to modulate the podocyte barrier properties through the induction of Kruppel-like factor 2 as a response to shear-stress[30,31]. A schematic of these interactions is presented on **Figure 1.4**.

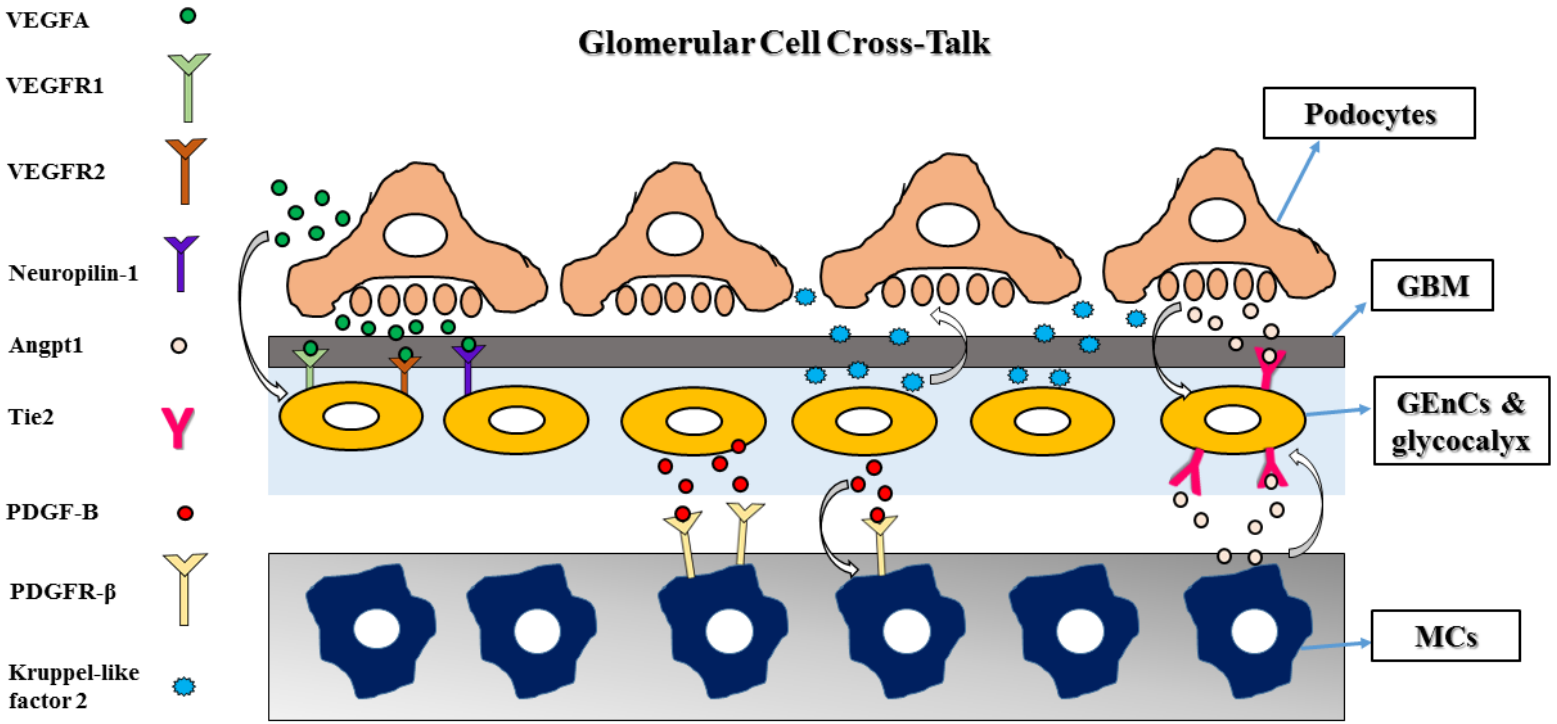


Figure 1.4 Schematic representation of glomerular cell interactions.

Glomerular cell crosstalk is achieved via several modes of interaction. The podocytes secrete VEGFA which can bind its cognate receptors VEGFR1, VEGFR2 and neuropilin-1, expressed by the GEnCs. Fluid laminar flow shear stress induces the production of the Kruppel-like factor 2 by the GEnCs that can modulate the podocyte barrier functions. Furthermore, the GEnCs can secrete PDGF-B that binds onto the MCs-expressed PDGFR-β and is involved in MC proliferation. Finally, both the podocytes and MCs secrete Ang-1 which, via binding to Tie2 expressed by the GEnCs, modulates the endothelium's barrier properties.

1.2. Systemic Lupus Erythematosus: A multifactorial autoimmune disease

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multiple clinical and immunological characteristics. A UK retrospective study from 1999 to 2012 was conducted using the Clinical Practice Research Datalink (CPRD), which provided evidence with regards to the incidence and prevalence of adult SLE[32]. This study demonstrated that SLE incidence (new cases every year) declined annually and was reported to be 4.91/100,000[32]. In contrast, SLE prevalence (number of cases in any given year) increased, perhaps because of improved disease management leading to significantly increased SLE patient survival, from 64.99/100 000 in 1999 to 97.04/100 000 in 2012, with women being predominantly affected[32]. SLE develops as a result of the combination of several confounding factors such as genetics, hormones and environmental factors. SLE pathogenesis involves aspects of both the innate and adaptive immune system[33–40]. SLE is the archetypal systemic autoimmune disease, characterised by multi-organ involvement, including the skin, blood, brain, lungs and kidneys[41,42]. A number of candidate genes, among them those encoding early components of the classical complement pathway such as C1q and C4[43], as well as genes involved in regulation of the interferon-alpha (IFN-α) pathway[44,45], have been defined as contributors to the onset of SLE. However, candidate genes themselves are not enough to initiate the disease alone. Furthermore, certain ethnic groups such as individuals of Black African or Asian heritage have a greater risk of developing SLE[46], potentially due to shared gene variations. Environmental agents such as a viral encounter, exposure to ultraviolet rays and certain medications (including IFN-α used for treatment of viral infections) can trigger the disease in a susceptible individual[47]. The complex interaction among the above-mentioned factors can lead to a variety of innate and adaptive immune responses. Some of the most important immunological

features of SLE include: increased production of anti-nuclear and anti-DNA autoantibodies by autoreactive B cells (the hallmark of SLE)[35], increased expression of type I IFN signature genes[37,38] and increased production of pro-inflammatory cytokines. Furthermore, increased neutrophil apoptosis and NETosis (NET: neutrophil extracellular trap) which, in combination with the imperfect phagocytosis observed in SLE, result in prolonged autoantigen exposure and breakdown of tolerance[33,34,39].

1.2.1. Comparison between adult- and juvenile-onset SLE.

SLE occurs less frequently in children compared to adults and data from different populations worldwide indicate that the incidence of juvenile-onset SLE (JSLE) is between 0.36–0.9 per 100,000 children per year[32,48–51]. Approximately 15-20% of SLE patients develop their first symptoms in childhood or adolescence (before 18 years old)[52,53]. JSLE is more common in girls, with the female:male ratio being approximately 4:3 before puberty suggesting a role for the X-chromosome; however, pubertal female:male ratio increases up to about 5:1[54] suggesting an important role for sex hormones and particularly oestrogens[54,55]. The sex differences increase even more in adulthood, with the female:male ratio being 9:1[52] . Higher incidence of JSLE has been reported in patients of Black African or Asian origin and these patients have earlier disease onset, generally more severe disease and increased nephritis incidence[46].

Data from studies where direct comparisons were made between adult-onset SLE (ASLE) and JSLE patients indicate that JSLE is generally more severe and aggressive and it presents with more acute disease features compared to ASLE[56,57]. There is also an associated increase in the medication burden (e.g. greater need for corticosteroid usage and immune-suppressive agents). Comparison of the clinical manifestations characteristic in adult-onset and childhood-onset disease has demonstrated that both

ASLE and JSLE patients have similar frequency of skin, joint, and central nervous system (CNS) damage[58]. However, ASLE patients are more frequently affected by cardiovascular disease, whereas in JSLE patients, haematological and renal complications are more commonly occurring[52,58].

In JSLE the levels of anti-double stranded DNA (anti-dsDNA) antibodies are higher compared to ASLE[59]. As with ASLE, changes in the levels of anti-dsDNA antibodies are monitored in order to determine disease activity in JSLE[60]. Children and adolescents generally have higher mortality rates directly attributable to SLE disease and suffer from greater disease damage than adults with SLE[52,56]. In both JSLE and ASLE, the major causes of death include renal disease, severe disease flares, and infections[61,62].

A study using data from the UK JSLE Cohort Study and Repository for both childhood-onset (diagnosis before 12 years old) and juvenile-onset (diagnosis between 12 and 17 years old) SLE as well as from the University College London Hospitals (UCLH) SLE cohort for both ASLE (diagnosis between 18 and 49 years old) and mature-onset (diagnosis after the age of 50 years old) SLE, assessed phenotypic and disease severity differences among different age groups [63]. The study demonstrated that lupus nephritis had a significantly higher prevalence in JSLE patients (44%) compared to both adult- and mature-onset SLE patients (33%) and confirmed female predominance, especially at puberty and adult ages. No age group differences were observed for patients with rashes or photosensitivity but JSLE patients were reported to be affected more by alopecia and oral ulcers whereas ASLE patients were more likely to be affected by arthritis and serositis. No significant differences were observed for neurological disease among age groups, however mature-onset SLE patients had

markedly lower presence of neurological involvement. JSLE patients had significantly higher immunological (anti-dsDNA antibodies, anti-RNP, anti-Sm autoantibodies) and haematological (thrombocytopenia and haemolytic anaemia) involvement, however leucopenia increased with advanced ages. This study supported the finding of previous studies that JSLE is more aggressive and with worse outcome than ASLE and although mortality rates have been decreasing during recent decades, they still are higher than the general population in both JSLE and ASLE [63].

In conclusion, from all the studies between JSLE and ASLE patients it is evident that although JSLE and ASLE share many common characteristics, they have also distinct features with different clinical phenotypes. Therefore, studying JSLE and ASLE as one continuum based just on age, and treating the JSLE patients just as "young ASLE patients", could potentially miss significant differences and invaluable insights into the pathophysiological mechanisms and potential therapeutic implications of childhood-onset disease. For this reason, it is vital to study both ASLE and JSLE, allowing for the development of more effective and targeted therapies.

1.3. Lupus Nephritis

In JSLE, between 50% and 75% of patients will develop kidney involvement[64] and 10 to 50% of these individuals will eventually progress to end-stage renal failure[65,66]. The most common clinical manifestations of lupus nephritis include: microscopic haematuria (79%), and proteinuria (55%)[67]. Decreased glomerular filtration rate (GFR) and hypertension are also observed (50% and 40%, respectively)[67].

Due to the severity and aggressiveness of LN, there is a pressing need to gain a much deeper understanding of the pathophysiological mechanisms underpinning the

development of lupus nephritis in order to identify better and potentially novel treatments and therapies.

1.3.1. Dysregulated apoptosis and imperfect cell clearance

SLE can develop in individuals bearing gene mutations which compromise those immunoregulatory mechanisms that under physiological conditions impede the presence of high levels of self-antigens (such as chromatin) in the extracellular spaces of the human body. Such mechanisms include: apoptosis,[68,69] complement opsonisation of dead cells, as well as effective phagocytosis of apoptotic cells[70]. In SLE imperfect phagocytosis of apoptotic cells leads to the increased presence of circulating apoptotic material, such as nucleosomes and cell membrane components[71]. These circulating autoantigens can potentially stimulate autoreactive T- and B cells, and promote immune complex formation[72].

Neutrophils are also able to undergo NETosis, which results in the release of nuclear material into the extracellular space (NETs) in order to trap extracellular pathogens and during homeostasis these would be cleared by incoming phagocytes[39,73]. However, in SLE delayed removal of dead cells due to imperfect phagocytosis leads to the sustained exposure of their components (e.g. nucleosomes and cell membrane components) to the immune system as well as to their gradual degradation[74,75]. This nuclear component degradation could lead to the loss of epigenetic modifications such as DNA and RNA methylation that normally aid the immune system to distinguish between self and foreign antigens and consequently trigger the induction of anti-viral immunity against autoantigens via type one interferon (IFN I: IFN-α and IFN-β) release[74–76]. In SLE, nuclear autoantigens are recognised as viral-like particles (for example U1snRNP ligation to TLR-7 or nucleosomal DNA ligation to TLR-9

expressed by pDCs). Plasmacytoid dendritic cells (pDCs) exhibiting a strong IFN type I gene signature, can initiate an anti-viral immune response, leading to SLE symptoms, namely fatigue, fever, myalgia and arthralgia[69,76–78].

1.3.2. Formation of immune deposits in LN

Lupus nephritis initiation is triggered by the production and deposition of anti-double-stranded DNA (dsDNA) antibodies (and other, less abundant autoantibodies) and/or the formation of *in situ* immune complexes in the renal parenchyma including the glomerulus, the vasculature, and the mesangium as well as the tubular interstitium of the kidney[79–82] .

In healthy individuals, anti-dsDNA autoantibodies of the IgM isotype class belong to the normal antibody (Ab) repertoire and display weak reactivity and low affinity for the self-antigens[83]. However, in SLE patients, these anti-dsDNA IgM antibodies undergo an isotype change to IgG class antibodies (probably due to increased presence of autoreactive B cells) with increased pathogenic potential, as they can fix complement by promoting the binding of the C1-complex to their Fc (fragment crystallisable) regions[84–86]. Furthermore, these pathogenic IgG autoantibodies display high affinity for self-antigens[87,88] as well as cross-reactivity with non-DNA self-antigens expressed on native kidney cells including α-actinin[89] , heparan sulfate proteoglycans[90,91] , laminin[91], collagen[91] and annexin II [92].

Apart from deposition of autoantibodies in the renal compartments, passive entrapment of preformed circulating immune complexes can occur in the glomerulus although this event is not the most significant mechanism of the formation of immune deposits in LN[93]. The imperfect clearance of apoptotic cells in lupus nephritis can lead to the release of nucleosomes from apoptotic intraglomerular cells[94] and subsequently

to nucleosome capture within the GBM where they serve as platforms for the indirect autoantibody binding to the GBM[95–97] .

Immune complex deposition can occur in different renal compartments (mesangium, subendothelial, subepithelial, and tubulointerstitial spaces) depending on the type of autoantibodies, the duration, and severity of LN[98,99]. Therefore, there is a clear association of histological classification and severity of LN renal injury with the presence of immune complexes in different renal spaces. Immune complex deposition in the mesangium is characteristic of 2003 International Society of Nephrology (ISN)/Renal Pathology Society (RPS) class I (minimal mesangial) and II (mesangial proliferative) glomerulonephritis (GN), subendothelial immune deposits are associated with class III (focal) and IV (diffuse) GN, and subepithelial immune complexes characterise class V (membranous) GN[98].

1.3.3. Activation of glomerular cells by immune deposits

Immune complexes can activate glomerular renal cells such as the MCs, by glomerular renal cell-expressed Fc receptor (FcR) ligation to the Fc regions of autoantibodies[100]. Furthermore, anti-DNA antibodies can activate endothelial and MCs through autoantibody internalisation by the renal cells following receptor binding[101,102].

There is evidence from *in vitro* studies that anti-dsDNA antibodies can bind to MCs, podocytes, endothelial cells, and proximal tubular cells[79,80,102,103] and that this event can promote functional changes in the intra-renal cells[80,104–107].

Anti-dsDNA antibodies can bind to the mesangium through attachment to chromatin[103] or via direct binding to cross-reactive antigens including α-actinin and annexin II[89,92,108]. The autoantibody binding promotes functional changes in MCs

including increased production of pro-inflammatory cytokines and pro-fibrotic mediators such as hyaluronan, interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α), transforming growth factor-beta 1 (TGF-β1), and fibronectin[92,109,110].

Glomerular endothelial cell activation and injury is a common event observed in severe proliferative lupus nephritis[98,111]. Activation of GEnCs leads to upregulated surface expression of adhesion molecules such as Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1)[112], which promotes stabilisation and adhesion of infiltrating leukocytes to the sub-endothelial and mesangial kidney regions and results in immune-mediated renal injury.

Incubation of human umbilical vein endothelial cells (HUVECs) with human polyclonal anti-dsDNA antibodies from lupus patients can stimulate the expression of VCAM-1, ICAM-1, and von Willebrand factor; these have been correlated with increased expression levels of the inflammatory cytokines IL-1, IL-6, and IL-8, which play an important role in lupus nephritis[105,106,113]. However, these data have not be validated using glomerular endothelial cells.

1.3.4. Chemokines, cytokines, soluble inflammatory mediators, and Toll-like receptors (TLRs)

Type I interferons (IFNs) play a significant role in the pathogenesis of lupus nephritis[114,115]. Both infiltrating and intra-renal immune cells as well as GEnCs can synthesise pro-inflammatory IFN alpha and gamma (IFN-α and IFN-γ)[116]. Increased levels of type I-IFNs (IFN-α and IFN-β) can stimulate dendritic cells which in turn can activate T- cells to cause tissue damage and B cells to produce more autoantibodies[117].

Immune complex deposition stimulates the production of numerous inflammatory mediators inside the nephritic kidneys, with the inflammatory mediator production being further enhanced as disease progresses[118,119]. CC-chemokine ligand 2 or monocyte chemoattractant protein-1 (CCL2 or MCP-1 respectively), is a chemokine expressed early in GN development, while tumor necrosis factor-α (TNF-α), is expressed upon initiation of proteinuria[120,121] suggesting a temporal release of cytokines is occurring. Nephritic kidneys are also characterised by a type I IFN signature which can cause vascular damage and PEC and podocyte injury[122–124]. The injured renal GEnCs release a number of soluble mediators such as nitric oxide (NO) which can damage podocyte foot processes, leading to podocyte loss[125].

Furthermore, the nucleic acids which are part of the immune complexes can promote intra-renal inflammation via immune complex endocytosis and nucleic acid recognition by toll-like receptors (TLRs) which are expressed by intra-renal macrophages and dendritic cells[126]. TLRs are involved in anti-microbial immunity[127] and therefore self-nucleic acids can be falsely recognised as foreign antigens (for example a virus), leading to an initiation of immune response against them.

1.3.5. Disruption of cell-cell communication among glomerular renal cells

Cell-cell interaction is of utmost importance for the maintenance of the GFB as well as of the nephron structure[128]. Direct contact of the GEnCs with the MCs ensures the protection of the GEnCs from transforming growth factor (TGF)-β-mediated damage, as the MCs sequester TGF-β on αvβ8 integrin expressed on the MC surface[129,130].

In LN as well as other renal pathologies, in both human renal diseases and animal models, expression of certain PDGF isoforms by MCs is upregulated, triggering

excessive proliferation of MCs, ECM deposition, cytokine and chemokine release, and renal fibrosis[28]. More specifically, abnormal PDGF isoform expression involves PDGF-A in rat models and human renal vascular rejection, PDGF-C and PDGF-B in rat models[131–134] and PDGF-C in human renal biopsies and rat models [131 135].

Loss of VEGFA which is necessary for GEnC survival and is secreted by podocytes is a feature of LN both in SLE patients but also in mouse models of the disease. Furthermore, affected GEnCs and podocytes in LN release endothelin-1 which aggravates glomerular injury by triggering a mitochondrial stress response[136]. Mature podocytes are terminally differentiated and have a limited proliferative capacity making regeneration and replacement of injured podocytes very difficult after cumulative damage in LN. This has as a consequence the development of glomerulosclerosis and loss of nephrons[137].

A schematic representation of the above-described events is presented on **Figure 1.5**.

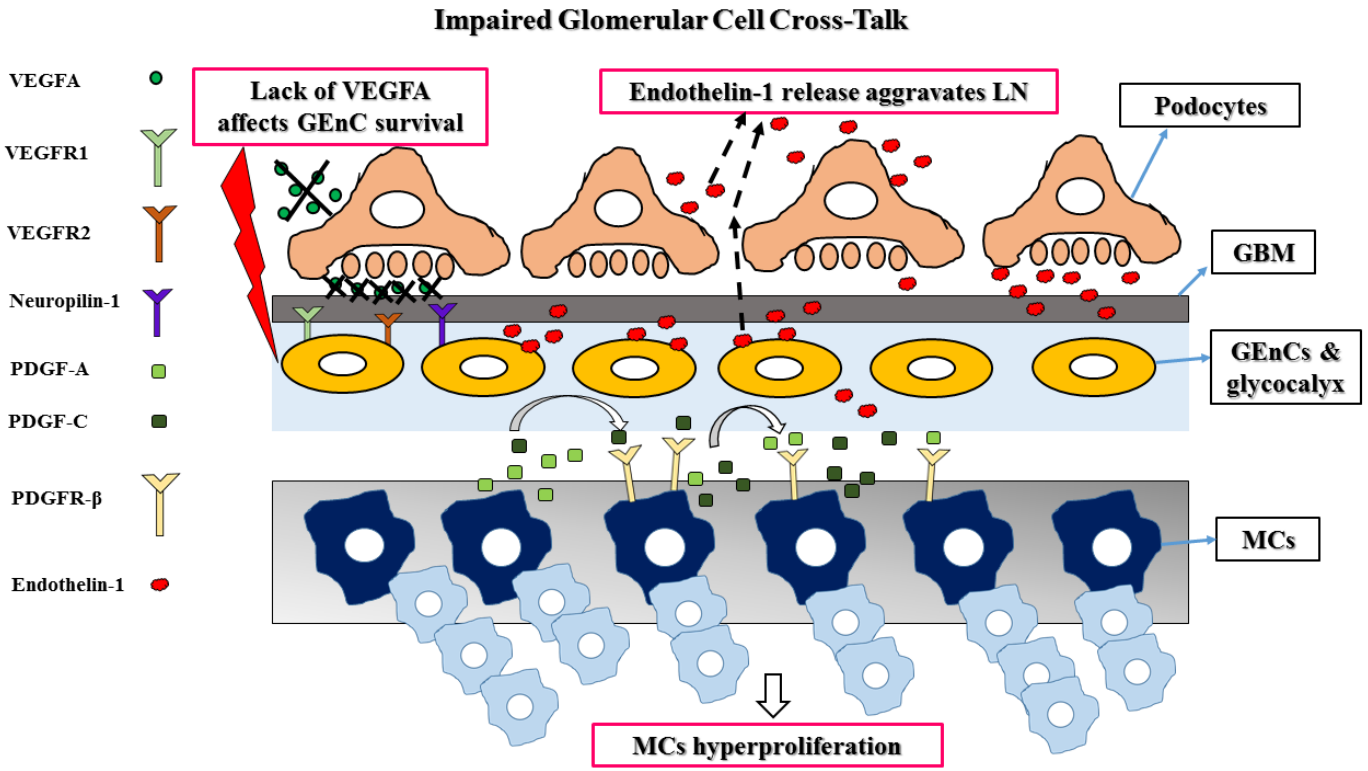


Figure 1.5 Schematic representation of impaired glomerular cell interactions in LN.

Upon renal disease, abnormal glomerular cell crosstalk can occur, leading to LN exacerbation. Different isoforms of PDGF can be produced in the glomerulus by the MCs leading to MC hyperproliferation, lack of VEGFA production can affect the survival of GEnCs whereas endothelin-1 production by podocytes and GEnCs can further aggravate the renal disease.

1.3.6. Chronic Kidney Disease (CKD)

If lupus nephritis is left untreated, or an abrupt decline in kidney function (leading to low GFR and to a rise of serum creatinine, known as acute kidney injury (AKI)), is not completely reversed, severe renal parenchyma injury occurs. Damage to renal cells initiates fibrotic processes such as increased extracellular matrix production and deposition by PECs and MCs and replacement of the renal parenchyma with fibrous tissue[138]. The accumulation of fibrotic tissue causes reduced renal function and thus develops into CKD which will require renal replacement therapy (dialysis or transplantation).

It is important to note that AKI and CKD are closely associated and interlinked, and can initiate one another, as accumulating evidence suggests that not only unresolved AKI can lead to CKD but also underlying CKD can promote AKI[139,140]. In LN, patients with AKI (that could be resulting from a renal flare) can suffer from significantly higher occurrence of thrombocytopenia and anemia, serositis and nephrotic syndrome, a condition characterised by heavy proteinuria, oedema and low albumin serum levels; they are furthermore characterised by decreased levels of serum C3 and by a poorer renal outcome[141]. In another study with a cohort of children with glomerulonephritis-related AKI, haematuria, heavy proteinuria and lipiduria are linked to AKI[142].

In contrast to AKI, which is commonly reversible, CKD is permanent, resulting in an irreversible decline in the number of functional nephrons and eventually leading to irreversible kidney failure[143]. In CKD caused by LN, renal damage is associated with proteinuria, haematuria, low GFR[143,144] and hypertension as well as an inability to achieve complete remission in the first year of treatment[145]. CKD can be categorised into 5 stages, from mild to moderate to severe CKD, with the latter requiring kidney replacement therapy[144]. Among the key molecular events contributing to CKD development, are inflammation involving -among

others- NF-κB activation and production/release of pro-inflammatory proteins at the sites of injury, renal fibrosis characterised by impaired scarring processes, extracellular matrix deposition and TGF-β-mediated epithelial-to-mesenchymal transition and accumulation of uremic toxins leading to endothelial cell dysfunction[146].

In LN, immune complex formation, abnormal function and TGF-β-mediated damage of the glomerular endothelial cells, TNF-α secretion at onset of proteinuria, kidney infiltration of macrophages, T cells (such as Th17 cells) and B cells and loss of intra-glomerular renal cell interactions as well as renal fibrosis are key contributors to CKD development[147].

1.3.7. Summary of LN pathogenic factors

LN pathogenesis involves both extra-renal and intra-renal factors. The extra-renal factors include specific combinations of genetic predeterminant variants for certain individuals that compromise immune tolerance for autoantigens as well as environmental factors and medications which lead to the production of anti-nuclear antibodies, the immunological hallmark of SLE. Furthermore, defective clearance of NETing or apoptotic neutrophils results in long-term exposure of nuclear particles that bind TLR-7 and TLR-9 and trigger a pseudo anti-viral immune response, orchestrated by IFN-α. As a result, the expansion of autoreactive dendritic cells, T helper cells, B cells and plasma cells is stimulated, and high levels of polyclonal autoantibodies are produced.

Renal damage in lupus nephritis begins with the formation of immune deposits in the kidney which trigger the activation of the complement cascades, influx of acute and chronic inflammatory cells inside the glomerulus, generation of inflammatory or fibrotic mediators from resident renal cells and infiltrating cells and activation of the coagulation cascade.

1.4. Pro-inflammatory proteins in lupus nephritis

Several studies have explored the differences in the expression of both systemic and intra-renal levels of pro-inflammatory factors and cytokines in patients with active LN[148–150]. Through these studies, it has become clear that LN patients exhibit a CD4+ T-helper (Th) 1 (Th1) cytokine profile (IFN-γ, TNF-α, IL-1β) [151 152]. However, specific CD4+ Th2 cytokines (IL-4, IL-13, IL-10) can also play an important role in LN.

Th1 and Th2 cytokines as well as pro-inflammatory molecules which have been shown to be involved in SLE and renal disease are presented below. The combined action of these molecules during renal flares or the action of different subsets of these pro-inflammatory factors during different stages of LN inflammation could lead to the promotion and sustainability of a highly inflammatory environment inside the nephritic kidney.

1.4.1. Tumour necrosis factor-α (TNF-α)

The role of TNF-α in LN is ambiguous as conflicting data have emerged from various studies.

Both protective [153] and detrimental [154] TNF-α effects have been reported in NZBxNZW and MRL-Fas^{lpr} lupus-prone mouse strains respectively, and the underlying mechanisms leading to this beneficial TNF-α response in NZBxNZW mice have not been clarified. For the NZBxNZW mice it was reported that complete TNF-α signalling depletion using anti-TNF-α Ab had deleterious effects on renal function whereas for the MRL-Fas^{lpr} mice, the administration of a TNF-α inhibitor or anti-TNF-α Ab reduced autoantibody production, TNF-α serum levels and inflammatory arthritis. Different studies have clearly demonstrated that NZBxNZW mice are characterised by TNF-α deficiency which drives lupus-like autoimmunity

[155–157]. On the other hand, in MRL-Fas^{lpr} mice TNF-α is found in abnormally high amounts in both serum and renal tissue and has been associated with the degree of inflammation in the diseased organs [158 159]. Nevertheless, even in NZBxNZW mice, when TNF-α was administered in low doses during later stages of disease progression, renal injury was accelerated [160]. These data could suggest that low, basal levels of TNF-α expression are necessary for a normal, healthy state but neither TNF-α overexpression nor total TNF-α ablation are protective against autoimmunity. Furthermore, the time of TNF-α administration, even in low doses, can play a crucial role in renal disease amelioration.

In human LN, serum levels of circulating TNF-α have been linked to glomerular protein expression of ICAM-1 which has been correlated with renal glomerular capillary damage in biopsy samples [161]. TNF-α gene polymorphisms leading to excessive TNF-α production can predispose patients to LN development and TNF-α protein levels have been found to be upregulated in the plasma of active lupus nephritis patients [162]. Additionally, in paediatric LN, podocyte cytoplasmic TNF-α overexpression has been reported [163]. A study comparing 60 serum samples from JSLE patients, found that TNF-α serum levels were significantly higher in patients with active LN compared to those without LN [164].

Results from two case studies have shown that patient TNF-α blockade treatment using infliximab (a chimeric monoclonal antibody targeting TNF-α) led to LN amelioration and improved long-term renal function for 9 out of 12 patients, [165] as well as mitigated proteinuria and improved SLE activity for 6 out of 8 patients [166]. However, other groups reported that anti-TNF-α treatments used in rheumatic diseases not only led to autoantibody production but also induced SLE as well as LN development [167 168]. Therefore, LN patients may be able to be categorised into ‘good’ and ‘bad’ anti-TNF-α responders, suggesting a potential application for

individualised anti-TNF-α therapy. However, it is currently unclear how patients should be stratified for this treatment.

1.4.2. Interleukin-6 (IL-6)

IL-6 is a cytokine with pleiotropic effects expressed by diverse cell types, including macrophages, dendritic cells, B cells and T cells as well as fibroblasts, mesangial and endothelial cells [169]. A number of pro-inflammatory factors such as interleukin-1 (IL-1), TNF-α and lipopolysaccharide (LPS) induce high amounts of IL-6 production by different cell types including fibroblasts, keratinocytes and epithelial cells [169 170]. Among the main IL-6 functions are B- and T cell differentiation as well as the stimulation of acute phase protein production by human hepatocytes [171].

In human LN, peripheral blood mononuclear cells (PBMCs) from patients produce elevated IL-6 levels compared to healthy control patients [172] and high amounts of urinary IL-6 have been correlated with diffuse (class IV) active LN [173]. Furthermore, in a pristane-induced murine model for lupus, lower levels of anti-dsDNA antibodies were observed in IL-6 deficient mice compared to wildtype mice [174] suggesting IL-6 might play an important role in renal flares [174]. Data from Phase I trials have provided evidence that a monoclonal high-affinity anti-IL-6 Ab, sirukumab, could be a promising treatment for both SLE and LN patients by regulating the levels of abnormal B- and T cell populations and by acting locally in the inflamed kidney, respectively [175].

1.4.3. Interferons

Th1 cells are potent producers of both IFN-α and IFN-γ [176]. IFN-α and IFN-γ have been correlated with the presence of anti-dsDNA antibodies as well as with proteinuria in SLE

whereas murine LN models have also exhibited preponderance of IFN-γ–producing Th1 cells compared TNF-α-producing Th1 cells and a shift towards Th1-type response was observed[177].

1.4.3.1.IFN-α

IFN-α is predominantly produced by plasmacytoid dendritic cells (pDCs) [178] and has several functions including induction of IFN-γ production, monocyte differentiation into antigen-presenting dendritic cells, activation of natural killer (NK) T cells and promotion of B cell differentiation, as well as initiation of apoptosis, leading to further accumulation and exposure of cellular debris to the immune system[117,179] . Circulating IFN-α levels have been correlated to serological activity of SLE [180] whereas studies using PBMCs from SLE patients have confirmed an association between IFN-α signature gene expression and disease activity [181] [182]. Furthermore, a local IFN-α signature has been detected in biopsies from LN patients [183]. A study using a new single-molecule array (Simoa) digital ELISA technology which is 5,000-fold more sensitive than commercial ELISAs [184] enabled for the first time the accurate measurement and direct quantification of IFN-α in the serum of patients with ASLE and JSLE (as well as patients with interferon abnormalities caused by other diseases, such as diabetes mellitus and type I monogenic interferonopathies) compared to healthy controls even in attomolar (fg/ml) concentrations. The ASLE patients in this study displayed a 250-fold increase and the JSLE patients displayed a 750-fold increase in serum IFN-α levels compared to healthy controls. This assay could assist clinicians towards better disease monitoring and diagnosis of patients suffering from aberrant type I IFN regulation.

1.4.3.2.IFN-γ

The effect of IFN-γ in SLE disease activity has been studied in several murine models showing that recombinant IFN-γ administration promoted disease advancement, an effect

abrogated by monoclonal Ab treatment against IFN-γ [185]. Furthermore, different models of IFN-γ-depleted mice exhibited reduced GN [186] and were protected from premature death [186] and renal disease [187].

Contradicting outcomes for the role of IFN-γ in SLE and LN have emerged from different clinical studies; IFN-γ serum levels have been associated with SLE activity and IFN-γ has been correlated with the severity of renal disease as it was shown to upregulate protein production of the co-stimulatory molecule CD40 by cultured MCs [188]. On the other hand, decreased IFN-γ protein production by PBMCs of patients with LN has been demonstrated [189]. However, another study provided evidence for IFN-γ upregulated glomerular expression in patients with segmental diffuse (class IV) LN but not global diffuse proliferative (Class IV) LN[150]. For the same patients, an association between IFN-γ expression and anti-dsDNA antibodies as well as serum complement levels was found. These data might suggest that IFN-γ might be mainly involved in LN and not in systemic SLE.

1.4.4. Interferon gamma-inducible protein 10 (IP-10)

IP-10 is a chemokine produced by IFN-γ-activated monocytes, fibroblasts, and endothelial cells. IP-10 mediates T cell accumulation to the inflamed tissues where it has been shown to have an anti-angiogenic effect [190] [191]. IP-10 serum levels have been found to be significantly upregulated in LN patients [192] and subsequent studies have highlighted urinary IP-10 mRNA as a reliable indicator of class IV (diffuse proliferative) LN as well as a potential biomarker for treatment effectiveness (lower IP-10 levels signified better response to treatments) [193].

1.4.5. Interleukin-10 (IL-10)

IL-10 is a pleiotropic cytokine, mainly produced by monocytes and Th2 cells. IL-10 has been known to have anti-inflammatory properties, however in SLE mouse models, IL-10 has been shown to promote anti-dsDNA autoantibody production from B cells which subsequently bind to the glomerular compartments promoting the development of GN [194], a phenomenon that suggests a potent role for IL-10 in B cell activation.

A human study demonstrated that unstimulated cultured PBMCs from SLE patients secreted 33-fold higher levels of IL-10 compared to controls. [195]. Furthermore, a human LN study reported a significant correlation between intra-glomerular IL-10 expression (as well as of IL-12, IL-18 and MCP-1 expression) and histological activity index[150].

1.4.6. Interleukin 1-beta (IL-1β)

IL-1β is a leukocyte-produced prototypical inflammatory cytokine. IL-1β has been shown to promote the expression of the adhesion molecules ICAM-1 and VCAM-1 in kidney, lung, heart, and brain endothelial cells [196] as well as B cell autoantibody production [197] in lupus-prone mice. Furthermore, in human LN, IL-1β has been detected in the renal tissue of patients with diffuse (class IV) GN whereas intra-renal IL-1β mRNA was predominantly synthesised by infiltrating macrophages and monocytes [198].

1.4.7. Interleukin-8 (IL-8)

IL-8 is a potent neutrophil chemokine, produced by monocytes and other cell types such as the endothelial cells where IL-8 is stored in the Weibel-Palade bodies (WPBs) [199 200]. In LN patients of African-American origin, a certain IL-8 gene polymorphism (IL-8-845C) which could potentially influence IL-8 expression (as hypothesised by the authors) has been

associated with more severe renal injury suggesting a potential for development of individualised immunosuppressive therapy [201].

1.4.8. Macrophage-Colony Stimulating Factor (M-CSF) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

M-CSF (or CSF-1) and GM-CSF (or CSF-2) are produced by various cell types including macrophages, lymphocytes, fibroblasts and endothelial cells [202] [203] [204]. GM-CSF promotes the maturation and differentiation of macrophages, neutrophils, eosinophils and basophils [205] whereas M-CSF mediates the growth, survival, differentiation and chemotactic activity of monocytes and macrophages [206].

In murine GN models the production of GM-CSF by both renal and immune cells was necessary for the development of renal glomerular injury [207] [208]. Furthermore, M-CSF–deficient lupus-prone mice exhibited amelioration of renal disease, suggesting an important role for M-CSF in lupus nephritis pathophysiology [209] [210]. In human LN, urinary M-CSF levels were found to be predictors of renal flares in diffuse (Class IV) GN patients [211] whereas serum and urine M-CSF could prove to be a reliable and sensitive biomarker for LN [212]. In addition, GM-CSF and M-CSF protein levels have been shown to be increased in human GN [213].

1.4.9. Macrophage Inflammatory Protein-1 alpha (MIP-1a)

MIP-1α (or CCL3) is a chemokine mainly produced by macrophages, dendritic cells, and lymphocytes [214] which activates neutrophils, eosinophils and basophils promoting acute neutrophilic inflammation. In human crescentic GN, MIP-1α together with MCP-1 has been shown to promote macrophage recruitment and activation in the kidneys [215]. Furthermore, in a human SLE study MIP-1α serum levels were strongly correlated with discoid lupus [216].

1.4.10. Interleukin-13 (IL-13)

IL-13 is a Th2 cytokine and is involved in allergic inflammation and IgE secretion by activated human B cells [217]. In SLE, IL-13 has been found in increased levels in the plasma, serum and PBMCs of patients with active LN [218] [219] [220]. In murine models of AKI, IL-13 has been shown to be necessary for the polarisation of the renal macrophages and dendritic cells towards an M2a phenotype (induced by IL-13 or IL-4 and characterised by macrophages TGF-β and arginase production), in order to achieve recovery from AKI [221].

1.4.11. VEGF

VEGF plays an important role in the preservation of glomerular endothelial cell functions, including their viability, growth, and permeability [222]. In childhood LN, plasma VEGF and its soluble receptor VEGFR1 were shown to be significantly increased in patients with active renal disease compared to patients in remission or healthy controls [223]. Therefore, although VEGF has an important beneficial role for endothelial cell survival, the increased plasma levels of VEGFR1 which binds VEGF with higher affinity than VEGFR2 (although it is VEGFR2 which mainly mediates the biological effects of VEGF)[224] sequesters VEGF from binding to VEGFR2 expressed on endothelial cell surfaces and initiates the signaling cascades leading to preservation of endothelial viability. In proliferative LN decreased intra-renal VEGF mRNA expression has been correlated with worse disease prognosis and deterioration of renal function[225].

1.5. From traditional to novel urinary LN biomarkers

The traditional clinical biomarkers used for LN monitoring such as measurement of proteinuria levels, the estimated GFR, blood pressure and hypertension as well as examination of the urinary sediment, have significant limitations in effectively identifying disease activity

in certain cases. Proteinuria can remain high for up to one year following renal flare while the urinary sediment (the deposit of cells derived from the centrifuged urine specimen which contains red cells, white cells or urinary casts in the urine collection) from patients with active LN could appear normal. It lacks specificity, whereas the monitoring of GFR has proved to be timely and costly and therefore unsuitable for routine use[226–228]. A summary of some of the advantages and limitations of traditional biomarkers for LN are shown in ***Table 1.1***.

Traditional Biomarkers	Advantages	Limitations	
Proteinuria (albumin to creatinine ratio)	Screening of renal involvement and prediction of renal outcome.	Poor specificity/Poor indicator of disease activity.	**These biomarkers are not specific for LN.**
Glomerular filtration rate (GFR)	Classification of chronic kidney disease.	Unsuitable for frequent JSLE monitoring due to high cost.	
Urinary sediment	Allows for microscopic evaluation/ Measurement of disease activity.	Cannot be solely used as a disease activity indicator.	

Table 1.1 Traditional urinary biomarkers, their advantages and limitations.

Several cross-sectional and longitudinal studies have taken place in order to identify easily accessible, non-invasive LN biomarkers in the urine of JSLE patients. Two novel urinary biomarkers, MCP-1 and neutrophil gelatinase associated lipocalin (NGAL), which were shown to distinguish between histological classes and predict deterioration of renal disease activity in adult disease, lacked specificity in JSLE[229–233].

Work from our group[234] as well as others[235] has provided strong evidence that the combination of four novel urinary biomarkers which are significantly increased in the urine of patients with LN are useful markers of disease activity in juvenile-onset LN. Those four biomarkers are: Alpha-1-acid glycoprotein (AGP), Lipocalin-type prostaglandin D synthase

(L-PGDS), transferrin (TF) and caeruloplasmin (CP), with AGP being the most consistent solitary indicator of renal flare.

Finally, urinary VCAM-1 has been shown to be significantly upregulated in JSLE patients with active lupus nephritis and therefore, it might be able to serve as an important indicator of renal disease activity[234,236].

1.5.1. Novel urinary biomarkers in LN

AGP is an acute phase plasma protein with drug-binding properties[237] mainly synthesised in the liver and secreted by hepatocytes. However, extra-hepatic production of AGP has been described: human breast epithelial cells as well as human microvascular endothelial cells (HMECs) can actively synthesise AGP[238,239].

The serum concentration of AGP increases rapidly several-fold upon inflammatory stimuli. AGP gene expression in hepatic tissues is regulated by the combined action of IL-1β, IL-6, TNF-α and glucocorticoids; this may also be important in extra-hepatic tissues[240].

AGP is a natural anti-inflammatory and immunomodulatory factor, due to its anti-neutrophil and anti-complement activity[241]; hence it might have a protective role for the kidney. Upon a LN flare, local and systemic upregulation of AGP could act as a signal against inflammation. Furthermore, circulating AGP could exert its anti-neutrophil and anti-complement properties in renal tissues, promoting the remission of flares.

L-PGDS is an enzyme which catalyzes the isomerisation of prostaglandin (PG) H2 (PGH2) to PGD2, which act as endogenous regulators of sleep and pain[242]. L-PGDS is mainly expressed in the brain, heart and male genital organs and is secreted in cerebrospinal fluid, plasma, and urine[242,243].

A study in which the localisation of L-PGDS in *Macaca fascicularis* or *Cynomolgus Macaques* primate kidney was examined by *in situ* hybridisation and immunohistochemistry, revealed that L-PGDS was synthesised *de novo* in the kidney[244].

TF is an iron-binding plasma glycoprotein that transports iron to all tissues and controls the free iron levels in biological fluids[245]. TF is principally synthesised in the liver and enters the cells through receptor-mediated endocytosis[245]. TF is an acute phase response protein. As kidneys express the TF receptor[246], TF uptake might be involved in the regulatory mechanisms of kidney homeostasis.

CP is a ferroxidase enzyme synthesised in the liver. It is the major copper-carrier in the blood (contains over 95% of the copper found in healthy plasma), and it also has a role in iron metabolism[247].

MCP-1 is a chemokine with the ability to promote kidney infiltration by inflammatory cells in lupus nephritis. MCP-1 is expressed by MCs, podocytes and monocytes in response to pro-inflammatory cytokines, such as TNF-α[248] . MCP-1 expression within the glomerulus can be predictive of poor renal outcomes in JSLE patients with lupus nephritis[229]. MCP-1 can also be found anchored in the surface of endothelial cells.

Neutrophil gelatinase-associated lipocalin (NGAL) is a member of the lipocalin family of proteins and plays a key role in the growth and differentiation mechanisms of renal tubular epithelial cells, and of epithelial cells in general[249]. It can also act as a bacteriostatic agent, by impeding the bacterial iron-acquisition mechanisms. NGAL has proved to be an efficient non-invasive biomarker as it is upregulated by the kidney in a number of acute renal injury conditions, such as urinary tract infection[250,251]. It has also displayed the ability to accurately predict renal flares in JSLE patients in cross-sectional cohort trials[252,253].

VCAM-1 is a cell-surface protein involved in the migration and adhesion of inflammatory cells such as lymphocytes, monocytes, eosinophils, and basophils to the vascular endothelium through which they can be recruited to inflammatory sites[254]. VCAM-1 promotes the recruitment and adhesion of inflammatory cells through its interaction with an integrin (very late antigen-4 or VLA-4) expressed by infiltrating leukocytes. VCAM-1 expression as well as VCAM-1 serum and urinary levels have been shown to be significantly upregulated in adult-onset SLE patients and especially, in LN[255,256]. Furthermore, in patients with active LN and more specifically Class III and Class IV GN, the levels of soluble VCAM-1 (sVCAM-1) were shown to be significantly elevated compared to LN patients in remission [257].

1.6. The glomerular endothelium in inflammation and biomarker production

As the role of the glomerular endothelial cells (GEnCs) in renal diseases has not been widely studied and limited data are available regarding their exact role in the renal inflammatory cascade, a general overview on the role of endothelial cells in inflammation is provided below.

Experiments presented here using human conditionally immortalised GEnCs (ciGEnCs) were designed based on both the general knowledge about the role of different endothelial cell types in inflammation (as described by previous studies and summarised below), but also according to research findings regarding the specific role of GEnCs in inflammation in general or in LN specifically. The human ciGEnCs were developed and kindly donated for our research by our collaborators at the University of Bristol, who used donated healthy human kidneys to isolate via differential sieving (described in more detail in section 2.2.2) primary human GEnCs, podocytes and MCs[258–260]. Using the same method for all three cell types, the primary GEnCs, podocytes and MCs were conditionally immortalised via a process that is described in more detail in section 2.2.2. Conditional immortalisation allowed for prolonged survival of the

conditionally immortalised GEnCs, podocytes and MCs in culture, overcoming the early senescence barrier of their primary counterparts, whilst also maintaining the same functional properties as the primary cells[258–260].

1.6.1. The endothelium under physiological conditions

Under homeostatic conditions the vascular endothelium performs specific basal functions, such as control of blood flow, maintenance of blood fluidity and coagulation inhibition as well as regulation of the capillary wall permeability and impeding leukocyte binding[261–266].

In non-inflamed tissues, the resting endothelial cells utilise different mechanisms to prevent interaction with leukocytes. Under physiological conditions endothelial cells express low levels of adhesion molecules including ICAM-1, VCAM-1 and E-selectin on their cell surfaces [266]. In addition, they store P-selectin as well as chemokines restricted inside secretory vesicles, the Weibel-Palade bodies (WPBs) ready for rapid release in response to injury or noxious stimuli [267] [199]. Furthermore, shear stress-induced nitric oxide (NO) production by the endothelial cells can suppress the expression of pro-inflammatory factors [268] and hinder the WPB fusion to the cell membrane [269] contributing to the overall leukocyte recruitment inhibitory environment.

Inability of the endothelial cells to exert these vital functions, leads to endothelial cell dysfunction, blood vessel compression and increased leukocyte binding onto the endothelium resulting in endothelial cell activation and initiation of the inflammatory cascade.

1.6.2. The stages of acute inflammation of the endothelium

Acute inflammation ensues within hours following a stimulus and is characterised by the recruitment of large numbers of neutrophils to the site of the activated endothelium.

Endothelial cell activation occurs in two stages, known as type I activation or stimulation and type II activation. Type I activation does not require new gene expression and leads to

rapid cell responses whereas type II activation necessitates new gene expression and therefore the cells elicit slower responses compared to type I activation [270]. Both types of activation result in local increases in blood flow, release of plasma fluid in the surrounding tissue and infiltration of circulating leukocytes which release mediators able to induce pain through interaction with specialised nerve fibres. Furthermore, factors released by the leukocytes can promote vasodilation and induce changes in the physiological functions of the resident cells of the invaded tissue by stimulating their activation.

1.6.2.1.Type I activation

Type I activation is induced by ligand binding, such as leukocyte-released histamine, to the extracellular part of G-protein-coupled receptor (GPCRs) histamine H1 receptors, that are expressed by the endothelial cells. [270] As a result, the endothelial cells are activated to secrete PGI_2 and produce nitric oxide (NO) [271] which synergistically promote vascular dilation leading to increased blood flow and enhanced circulating immune cell recruitment. Furthermore, endothelial cell cytoskeleton reformation can occur, leading to creation of gaps in the tight junctions between neighbouring endothelial cells which, in turn, allow for plasma protein leakage [272] and circulating neutrophil [273] and monocyte transmigration through interactions between platelet-endothelial cell adhesion molecule-1 (PECAM-1; also known as cluster of differentiation 31;CD31) expressed on endothelial cells and CD99 expressed on circulating leukocytes [274]. In addition, increased WPB exocytosis can result in P-selectin emersion to the luminal surface of the endothelium [275]; P-selectin, in turn, can promote the recruitment and tethering of both neutrophils and lymphocytes to the endothelial cells.

Type I activation does not require new gene expression and it approximately lasts for 10-20 minutes [276]. After their initial activation, GPCRs become desensitised in order to prevent continuous inflammation and further neutrophil and leukocyte recruitment [276].

Another example of Type I activation commonly observed in LN, is the glomerular invasion of pathogenic microorganisms such as bacteria, through the glomerular capillaries. There, substances released by the bacteria or expressed in the bacterial wall (for example LPS) could activate resident and/or infiltrating macrophages, which in turn, would secrete cytokines and chemokines leading to vasolidation, changes in GEnC permeability, PGI_2 secretion and P-selectin emergence to cell surface.

1.6.2.2.Type II activation

Type II activation requires the induction of gene expression and therefore promotes the formation of a more persistent inflammatory microenvironment. The prototypic pro-inflammatory cytokines orchestrating this response are TNF-α and IL‑1β which are produced by activated leukocytes [270].

TNF-α and IL-1β bind to their receptors expressed by the endothelial cells (TNFR-1/TNFR-2 and IL1-RI respectively) and initiate different cascade pathways which all converge at the activation of the nuclear factor-κB (NF-κB) transcription factor [277 278 279]. Furthermore, signaling through TNFR-1 and IL1-RI leads to the activation of another transcription factor, activator protein-1 (AP-1). NF-κB is heavily involved in the overall pro-inflammatory response of the endothelial cells by inducing the expression of multiple downstream target genes responsible for neutrophil, monocyte, macrophage and T cell recruitment and binding, including; IL-6, IL-8, IL-1β, RANTES, MCP-1, MIP-1α, IP-10, GM-CSF, M-CSF, IFN-γ, TNF-α, VCAM-1, ICAM-1, E-selectin and P-selectin[280,281]. In addition, the endothelial cells produce higher levels of PGI_2, due to cyclooxygenase-2 (COX-2) expression via NF-κB activation [280]. Both TNF-α and IL-1β can promote changes in the endothelial cytoskeleton through reorganisation of the actin and tubulin structures, resulting in gap formation between neighbouring cells and plasma protein leakage [282 283].

Type II endothelial activation is characterised by a temporal switch from the expression of the adhesion molecule E-selectin to the expression of the adhesion molecules VCAM-1 and ICAM-1 and of the chemokine MCP-1 which allow the gradual predominance of a mononuclear cell infiltrating population over the initial neutrophil accumulation in a time interval of 6-24 hours after the endothelial stimulation by TNF-α and IL-1β [284 285]. Factors produced by the leukocytes will eventually cause endothelial cell injury and plasma leakage through the capillaries, therefore enhancing the inflammatory response [286]. In addition, TNF-α and IL-1β in combination with IFN-γ (a cytokine known to be prevalent in LN) can further aggravate endothelial cell injury by inducing apoptosis[287].

1.6.3. Chronic inflammation

Failure of resolution of acute inflammation can lead to a more chronic adaptive immune response, where specialised effector T- and B- cells become key inflammatory players. The endothelial cells have been hypothesised to be involved in this process by antigen presentation to the circulating effector and memory T cells[288–290]. Furthermore, under chronic inflammatory conditions the endothelial cells can support the sustainability of either a $CD4^+$ Th1 or Th2 cell response, by differential production of chemokines and adhesion molecules[291,292].

Prolonged periods of inflammation without resolution through effector cell action subsequently lead to angiogenesis; the endothelial cells migrate to the surrounding tissues and new capillary networks are generated in order to sustain the viability of the accumulated inflammatory cells[293], as observed in both rheumatoid arthritis (RA) and atherosclerosis[294].

Finally, chronically inflamed tissues can promote the formation of tertiary lymphoid organs (TLOs) which consist of naïve and memory T cells as well as B cells[295]. The endothelial cells support the sustainability of the TLO-structures by changing their shape as well as by expressing L-selectin ligands which mediate the recruitment of the TLO-associated lymphocytes[296].

Often, the mediators of acute and chronic inflammation can have overlapping activities; therefore, it might not be simple to distinguish between the two processes. For instance, TNF-α signaling through TNFR-1 and TNFR-2 can induce type II acute inflammatory responses via NF-κB activation but TNFR-2 signaling can also have a pro-angiogenic effect, indicative of chronic inflammation, through vascular endothelial growth receptor factor receptor-2 (VEGFR2) activation[297].

1.6.4. The glomerular endothelium in inflammation

Biopsies of LN patients have been found to exhibit upregulated protein expression and activation levels of NF-κB in GEnCs (and also MCs), which correlated with increased production of pro-inflammatory cytokines and cell adhesion molecules such as TNF-α, IL-1β, IL-6 and ICAM-1 [298] [299].

Furthermore, IL-1β treatment was shown to promote permeability in cultured primary human GEnCs [300], an effect that could lead to GFB disruption and subsequent increase in proteinuria levels whereas kidney injury induced by LPS and TNF-α was shown to promote renal heparenase expression and subsequent damage of the GEnCs' ESL in a mouse model of AKI sepsis [301]. In both human and murine cultured GEnCs, TNF-α was also shown to increase albumin permeability, alter the size of fenestrations, induce changes in the actin cytoskeleton, create gaps between adjacent GEnCs, decrease the levels of occludin (a tight junction protein) and cause glycocalyx degradation, via the Rho and myosin light chain kinase (MLCK) signalling pathway [302].

Therefore, NF-κB, together with TNF-α and IL-1β, seems to have a prominent role not only generally in endothelial cell inflammation but also specifically in GEnC inflammation.

1.6.5. Glomerular endothelial cell function and role in biomarker expression

As the GEnCs line the interior surface of the glomerular capillaries, they come in direct contact with circulating blood and serum components in the lumen. Therefore, under renal pathogenic situations such as in lupus nephritis, the formation of a pro-inflammatory microenvironment (e.g. cytokine expression by circulating inflammatory cells) in the glomerular capillaries, will eventually promote the increased expression of adhesion molecules on the surface of the GEnC. The increased expression of adhesion molecules will in turn lead to increased binding of leukocytes to endothelial cells and initiation of leukocyte kidney infiltration[112] .

Inflamed endothelial cells such as cytokine-activated HUVECs, express both E-selectin and P-selectin, which promote leukocyte rolling and binding to the vascular endothelium via glycosylated ligands[303,304] . Subsequently, leukocyte arrest and stable binding to the vascular endothelium are mediated through interaction of leukocyte integrins with immunoglobulin superfamily members expressed by the endothelial cells, such as ICAM-1 and VCAM-1[305,306].

Several prototypical cytokines, among them TNF-α, IL-1β, IL-4 and IFN-γ have been shown to upregulate the expression of adhesion molecules on vascular endothelial cells in culture and thereby to increase the binding of leukocytes[258,307–309].

Under the highly inflammatory environment of LN, the activated endothelial cells might produce elevated levels of biomarkers including AGP, L-PGDS, TF, CP, MCP-1, NGAL and VCAM-1 (but also ICAM-1) which, due to renal tissue injury, will be subsequently released in the urine.

1.7. Summary

Unraveling the mechanisms underpinning lupus nephritis inflammation and renal damage is of utmost importance in order to develop new, more effective treatments and improve the quality of life of JSLE patients. There is a great need not only to investigate the changes occurring in the structure and function of the GFB in lupus nephritis, but also find novel non-invasive biomarkers with high specificity and sensitivity for early LN diagnosis and monitoring. Although kidney biopsy is the gold standard for investigation of renal involvement and diagnosis of LN, it is an invasive and painful procedure, especially as some JSLE patients require a general anaesthetic.

Therefore, studying the role of the resident renal cells in the development of inflammation in LN could open new avenues for the development of novel and more targeted therapeutic approaches. The human GEnCs are an integral GFB component and at the same time are located at the interface between the blood microcirculation and the glomerular renal tissue. They could therefore be involved in the perpetuation of renal inflammation in LN through several different mechanisms including structural and functional changes (such as accelerated apoptosis), activation by the blood plasma and serum components and by pro-inflammatory cytokines. Furthermore, they could be actively involved in the production of the novel urinary biomarkers, secretion of pro-inflammatory chemokines and cytokines and surface expression of adhesion molecules and receptors, aberrant interactions with infiltrating and resident immune cells as well as with the podocytes and the MCs.

In this project, the human GEnCs were tested for their ability to produce various pro-inflammatory proteins under appropriate stimuli. These proteins and their involvement in lupus and lupus renal disease are presented below, in Table 1. X.

Proteins induced by TNF-α, IL-1β, IL-13, IFN-γ and LPS	Involvement in SLE and renal disease
MCP-1	MCP-1 expression within the glomerulus can be predictive of poor outcomes in JSLE patients with LN[229].
VCAM-1	VCAM-1 serum and urinary levels have been shown to be upregulated and associated with active LN[255,257].
ICAM-1	ICAM-1 urinary levels are upregulated in with class III, IV and V LN[310].
IL-6	High IL-6 levels have been detected in the urine of active LN patients[311].
IL-8	High IL-8 levels have been detected in the urine of active LN patients[311].
IL-10	Plasma IL-10 has been found to be significantly elevated in JSLE patients with active disease[312].
M-CSF	Serum and urine M-CSF levels have been shown to be a reliable and sensitive biomarker for LN[211,212].
GM-CSF	Increased mRNA levels of GM-CSF are expressed by in vitro cultured human renal tubular cells derived from interstitial fibrotic kidneys compared to tubular cells derived from non-fibrotic kidneys[313].
MIP-1α	Together with MCP-1, promotes macrophage recruitment and activation in the kidneys of patients with crescentic glomerulonephritis[215].
IP-10	IP-10 serum levels are upregulated in LN patients[192].
TNF-α	TNF-α gene polymorphisms leading to excessive serum TNF-α production can predispose patients to LN development[314].
IFN-γ	Significantly increased IFN-γ positive immune-histochemical staining has been observed in biopsies from juvenile-onset LN patients compared to HCs[315].
PD-L1	In JSLE, decreased PD-L1 expression by antigen-presenting cells has been associated with active disease[316].
ICOS-L	ICOS-L plasma levels are increased in patients with active SLE compared to those with inactive SLE[317].

Table 1.2 Proteins produced by activated ciGEnCs and involvement in renal disease.

1.8. Scope of the thesis

1.8.1. Hypothesis

The human GEnCs are not just passive bystanders but are actively involved in the perpetuation of renal inflammation in LN.

1.8.2. Aims

The hypothesis will be addressed using the following aims and objectives (**Figure 1.6**):

- **Aim-1**: To assess the pro-inflammatory profile of the human GEnCs using an *in vitro* model of human ciGEnCs **(Chapter 3)**.

-Objective 1: To determine the effects of inflammatory cytokines involved in LN in ciGEnC expression of novel urinary biomarkers for LN and pro-inflammatory protein production, and to determine which of these cytokines have the most prominent effect.

-O**bjective 2:** To test whether the pro-inflammatory stimulated ciGEnCs activate important pro-inflammatory transcription factors.

-Objective 3: To assess the viability of ciGEnCs after pro-inflammatory stimulation.

- **Aim-2:** To investigate the effect of the glomerular renal microenvironment on GEnC pro-inflammatory profile using multiple *in vitro* ciGEnC models **(Chapter 4)**.

-Objective 1: To assess whether ciGEnC treatments with JSLE patient and paediatric healthy control sera can modify their fibrotic and pro-inflammatory phenotype.

-O**bjective 2:** To determine whether glomerulus-infiltrating immune cells such neutrophils and macrophages could potentially have a paracrine effect on the pro-inflammatory activation of GEnCs.

-O**bjective 3:** To determine whether GEnC-neighbouring renal cells, podocytes and MCs have a paracrine effect on the pro-inflammatory activation of GEnCs.

- **Aim-3:** To investigate the effect of the novel urinary biomarkers for LN on GEnC pro-inflammatory profile using an *in vitro* ciGEnC model **(Chapter 5)**.

-Objective 1: To assess whether treatments with human recombinant urinary biomarker could promote the expression and/or production of novel urinary biomarkers for LN and pro-inflammatory proteins.

-O**bjective 2:** To determine whether the human recombinant urinary biomarker treatments can affect the ciGEnC viability.

Figure 1.6 summarises the approaches that will be taken to investigate the role of GEnCs in the development and perpetuation of inflammation in LN.

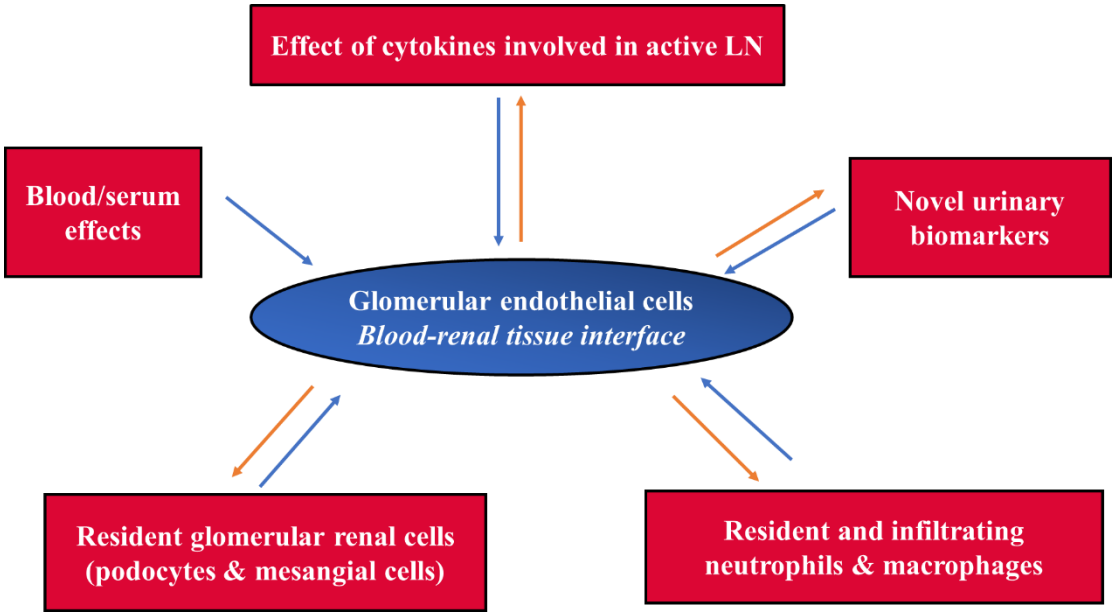


Figure 1.6 Summary diagram for the study of the role of human GEnCs in the development and perpetuation of inflammation in LN.

2. Chapter 2: Materials and Methods

This chapter provides an overview of key methodological techniques used for the design and development of experimental assays used to address this thesis' key hypothesis, aims and main objectives (see section 1.8).

2.1. Materials

The specific materials used in the different methodological assays are summarised below.

2.1.1. Whole blood processing

In order to process blood samples, HetaSep, an erythrocyte aggregation agent aiding separation of red blood cells from white blood cells, was purchased from StemCell whereas Histopaque 1077, a density gradient cell separation Ficoll-based medium aiding separation of neutrophils from PBMCs, was purchased from Sigma-Aldrich. A red blood cell lysis buffer containing ammonium chloride, potassium carbonate and ethylenediaminetetraacetic acid (EDTA) was also used (Sigma-Aldrich).

2.1.2. Cell culture

All cell types were washed using Phosphate Buffered Saline (PBS, Sigma-Aldrich). For the ciGEnCs 0.25% trypsin (59428C, Sigma-Aldrich) was used. Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich (A9418).

Human macrophages and neutrophils isolated from whole-blood samples with the use of HetaSep and Histopaque were cultured in Roswell Park Memorial Institute (RPMI) medium-1640 (RPMI-1640) that was purchased from Sigma-Aldrich, with 10% Fetal Bovine Serum (FBS), purchased from Gibco. Human recombinant M-CSF (R&D Systems, 216-MC) was used for monocyte differentiation into macrophages. For the culture of THP-1 cells RPMI medium 1649-GlutaMAX and L-glutamine were purchased from Gibco and antibiotic/antimycotic was

purchased from Sigma-Aldrich. Phorbol 12-myristate 13-acetate (PMA) was purchase from Abcam (ab120297).

For the culture of the human ciGEnCs, Endothelial growth medium 2-microvascular was purchased from two companies; Lonza (EGM2-MV Bulletkit, CC-3202) and PromoCell (MV-2 media kit, C-22121). For the culture of human ci podocytes and MCs RPMI-1640 (+L-glutamine, Lonza) with 10% FBS and 1% insulin transferrin selenium (ITS, Sigma-Aldrich) was used.

When necessary, cells were frozen using Dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich (D2650) and FBS.

2.1.3. Cytokines and lipopolysaccharide (LPS) used in assays

The human recombinant cytokines and the bacterial LPS used in this study, and their source, are presented on **Table 2.1** below:

Human Recombinant Cytokine	Catalogue Number	Company
Interferon-alpha (IFN-α)	300-02A	Peprotech
Interferon-gamma (IFN-γ)	300-02	Peprotech
Tumour necrosis factor-alpha (TNF-α)	300-01A	Peprotech
Interleukin-1 beta (IL-1β)	200-01B	Peprotech
Interleukin-6 (IL-6)	200-06	Peprotech
Interleukin-13 (IL-13)	200-13	Peprotech
Vascular endothelial growth factor (VEGF)	293-VE	R&D Systems
Lipopolysaccharide (LPS)	L2654	Sigma-Aldrich

Table 2.1 Cytokines and LPS used for treatments in this study.

2.1.4. Human recombinant novel urinary biomarkers investigated

The human recombinant biomarkers used for ciGEnC treatments, and their source, are presented on **Table 2.2** below:

Biomarker	Catalogue Number	Company
Human Recombinant MCP-1	300-04	Peprotech
Human Recombinant L-PGDS	NBP1-51078	Novus Biologicals
Human holo-Transferrin	2914-HT	R&D Systems
Natural human AGP	ab168874	Abcam
Human CP, purified from plasma	ABIN956559	Antibodies-Online
Human recombinant NGAL	1757-LC	R&D Systems

Table 2.2 List of the human urinary biomarkers used for ciGEnC treatments.

2.1.5. RNA extraction, cDNA conversion and SYBR-Green real-time PCR kits used

In order to undertake RNA extraction, the Qiagen RNeasy Mini Kit was used and Trizol reagent was purchased from Invitrogen. RNase-free DNase sets (79254) and nuclease-free water were purchased from Qiagen. cDNA synthesis and qRT-PCR kits from Agilent and Primerdesign were utilised for all experiments.

2.1.6. Antibodies used for flow cytometry

The antibodies and isotype control antibodies utilised for the detection of the adhesion molecules as well as the isotype control antibodies which were included in all experimental repeats are summarised on **Tables 2.3** and **2.4**.

Primary Antibody (anti-human) / Clone	Working Concentration	Catalogue Number, Company
VCAM-1 – FITC (Mouse IgG1, kappa)/STA	4 µg/ml	11-1069, eBioscience/Affymetrix
ICAM-1 – APC (Mouse IgG1, kappa)/HA58	1 µg/ml	17-0549, eBioscience/Affymetrix
E-/P-selectin – PE (Mouse IgG1)/BBIG-E6 (13D5)	4 µg/ml	FAB6169P, R&D Systems/Bio-Techne
PD-L1 – PE (Mouse IgG1, kappa)/MIH1	2 µg/ml	12-5983-42, Invitrogen/Thermo Fisher Scientific
ICOS-L-PE (Mouse IgG1, kappa)/9F.8A4	2 µg/ml	329806, BioLegend

Table 2.3 List of antibodies for FC.

Isotype Control Antibody	Clone	Catalogue Number, Company
Mouse IgG1 K- FITC	P3.6.2.8.1	17-4714, eBioscience/Affymetrix
Mouse IgG1 K- APC	P3.6.2.8.1	17-4714, eBioscience/Affymetrix
Mouse IgG1 K - PE	P3.6.2.8.1	14-4714-82, Invitrogen/Thermo Fisher Scientific/eBioscience
Mouse IgG1 - PE	M1-15D12	12-4016-82, eBioscience/Affymetrix

Table 2.4 Isotype control antibodies.

2.1.7. Materials used for flow cytometry-based assessment of apoptosis/necrosis

The apoptosis kit utilised was purchased from Sigma-Aldrich (APOAF). Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) was used to wash and re-suspend cells prior to flow cytometric analysis.

2.1.8. Materials used for enzyme-linked immunosorbent assay (ELISA)

R&D Systems DuoSet ELISA kits were utilised to identify potential treatment-induced changes in the secretion levels of human TNF-α (DY210) and IL-1β (DY201) by THP-1 cells and of human MCP-1 (DY279) and sVCAM-1 (DY809) by ciGEnCs.

A custom-synthesised Magnetic Luminex multiplex ELISA custom kit (LXSAHM) was purchased from R&D Systems and was used to detect 24-hour cytokine- and LPS-induced differences in the secretion levels by ciGEnCs of the following pro-inflammatory proteins; macrophage MIP-1α, IP-10, IL-6, IL-8, GM-CSF, M-CSF, TNF-α and IFN-γ.

2.1.9. Materials used for Western blotting (WB)

RIPA buffer (R0278) and protease inhibitor cocktail (P8340) were purchased from Sigma-Aldrich. Pierce™ BCA (Bicinchoninic acid) Protein Assay Kit (23225) was purchased from Thermo Fisher Scientific. 4x NuPAGE LDS Sample buffer was purchased from Invitrogen and dithiothreitol (DTT) from Sigma-Aldrich. Bio-Rad precast SDS-PAGE gels (4561044) and Bio-Rad vertical electrophoresis cells were used. 10x Running buffer was prepared as follows; 25 mM Tris-Base (Sigma-Aldrich), 190 mM glycine (Sigma-Aldrich), 0.1% SDS (Sigma-Aldrich).

Sample buffer at a final 1x concentration with 50μM DTT, as a reducing agent. The proteins were boiled for 5 minutes at 90^{o}C and were loaded in 12% 50μl 10-well precast SDS-PAGE gels (4561044, Bio-Rad) and protein gel electrophoresis took place into vertical electrophoresis cells (Bio-Rad) filled with 1x running buffer prepared from water-diluted 10x running buffer (25 mM Tris-Base, 190 mM glycine, 0.1% SDS). Polyvinylidene difluoride (PVDF) membranes and the Trans-Blot Turbo Transfer System from Bio-Rad Gels were used. 5% non-fat dry milk from Marvel was used. 10x Tris-buffered saline (TBS) with Tween-20

(TBS-T) stock solution (pH: 7.5) was prepared as follows: 100 mM Tris-Base (Sigma-Aldrich), 1.5 M NaCl (Sigma-Aldrich), 0.1 % Tween-20 (Sigma-Aldrich), 500 ml of ddH_20. Enhanced chemiluminescence (ECL) reagents (WesternSure ECL Substrate, 926-80100) for protein detection were purchased from Li-COR. Li-COR software was used for protein band densitometry. Mild stripping buffer (pH:2.2) was prepared as follows; 200 mM glycine (Sigma-Aldrich), 3.5 mM SDS (Sigma-Aldrich), 0.01 % Tween-20, (Sigma-Aldrich) and 500 ml of ddH_20.

The primary antibodies and secondary antibodies utilised for the assays are shown on **Table 2.5**.

Primary Antibody (anti-human)	**Working Concentration**	**Catalogue Number, Company**
Rabbit IκBα (monoclonal)	100 ng/ml	4812, CST
Mouse beta-actin	333.3 ng/ml	ab8226, Abcam
Secondary Antibody	**Working Concentration**	**Catalogue Number, Company**
Goat anti-mouse IgG-HRP	20 ng/ml	HAF007, R&D Systems
Goat anti-rabbit IgG-HRP	20 ng/ml	HAF008, R&D Systems

Table 2.5 WB primary and secondary antibodies.

2.1.10. Materials used for immunofluorescence (IF) assays

Formaldehyde solution and Triton-X-100 were purchased from Sigma-Aldrich. DAPI (4',6-diamidino-2-phenylindole) was purchased from Thermo Fisher Scientific (D1306).

Primary and secondary antibodies utilised for IF in this study are presented on **Table 2.6**.

Primary Antibody (anti-human)/Clone	Working Concentration	Catalogue Number, Company
Rabbit RelA/NF-κB p65/Polyclonal	400 ng/ml	NB100-2176, Novus Biologicals/Bio-Techne
Mouse STAT-1/655210	780 ng/ml	MAB14901, R&D Systems/Bio-Techne
Rabbit STAT-2/Polyclonal	1.25 µg/ml	44-362G, Thermo Fisher Scientific
Secondary Antibody	**Working Concentration**	**Catalogue Number, Company**
Goat anti-mouse - A568/Polyclonal	4 µg/ml	A-11004, Thermo Fisher Scientific
Goat anti-rabbit - A488/Polyclonal	4 µg/ml	A-11008, Thermo Fisher Scientific

Table 2.6 IF primary and secondary antibodies.

2.2. Methods

All methods reported here were carried out in accordance with relevant guidelines and regulations of the University of Liverpool. All experimental protocols were reviewed and approved by either the North West – Liverpool East Research Ethics Committee (UK JSLE Cohort Study and Repository) (REC: 06/Q1502/77) or the University of Liverpool Committee of Research Ethics (Adult Healthy Control samples) (Research Ethics Approval Number: RETH000773). Paediatric healthy control whole-blood samples were obtained pre-surgery, from young patients being admitted to Alder Hey Children's Hospital to undergo non-emergency surgery and who, at the time of the sample acquisition, presented with no autoimmune or inflammatory conditions that could affect experimental results. Informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian.

2.2.1. Isolation of neutrophils and PBMCs from whole blood

Human blood was obtained from adult healthy volunteers using a 21-gauge needle and transferred to a collection tube containing heparin-coated beads to prevent clotting. The blood

was transferred to a 10mL tube and immediately HetaSep was added to the blood at a volume 5-fold lower than that of the whole blood. Whole blood and HetaSep were gently mixed and left at room temperature for 30 minutes for erythrocyte sedimentation to occur (**Figure 2.1**). The top plasma layer containing neutrophils and Peripheral Blood Mononuclear Cells (PBMCs: lymphocytes, monocytes and dendritic cells) was collected, placed in a new tube and diluted with sterile PBS at a volume 4-fold higher than that of the plasma layer. The PBS-plasma solution was centrifuged at 200 xg for 10 minutes. The supernatant was discarded and the cell pellet consisting mainly of leukocytes (neutrophils and PBMCs) was re-suspended in 1 ml of RPMI 1640 medium. Subsequently, this was layered onto 2 ml of Histopaque in a new 10 ml tube and centrifuged at 300 xg for 20 minutes, leading to neutrophil and PMBC separation, with neutrophils forming a pellet on the bottom of the tube and PBMCs forming a cloudy ring at the interface between Histopaque and RPMI **(Figure 2.1)**. The PBMCs were collected, placed into a new 10 ml tube and diluted with PBS. The neutrophil pellet was re-suspended in 1 ml of RPMI and 9 ml of 1x red blood cell lysis buffer (containing 155mM ammonium chloride, 10 mM potassium carbonate and 0.1 mM EDTA). This was gently inverted for 3 minutes at room temperature to ensure all erythrocytes were lysed. Finally, the PBMC- and neutrophil- suspensions were pelleted by centrifuging for 5 minutes at 300 xg and PBMCs and neutrophils were utilised for downstream experiments.

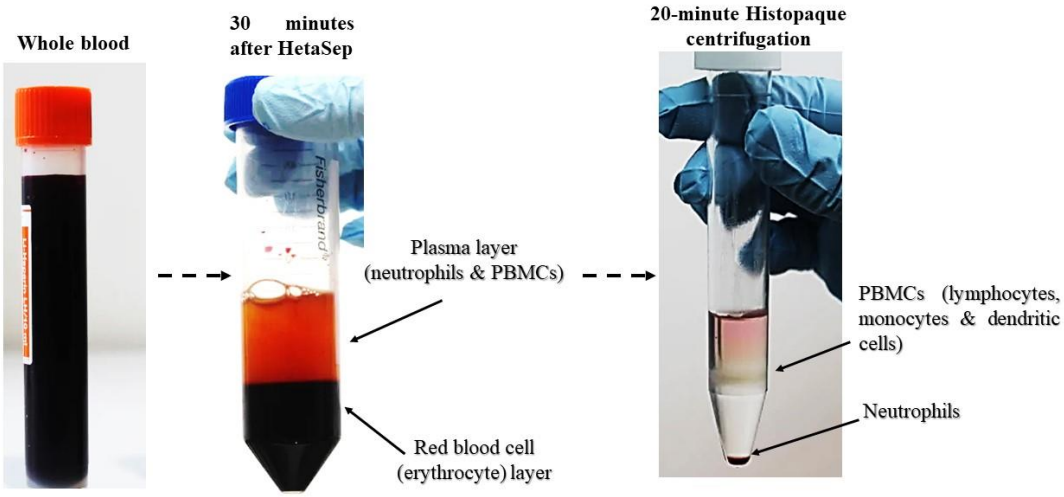


Figure 2.1 Neutrophil and PBMC isolation from whole blood.

HetaSep is initially added to whole blood to separate leukocytes from red blood cells. Leukocytes, present on the plasma layer, are then separated into neutrophils and PBMCs via Histopaque sedimentation.

2.2.1.1. Primary macrophage culture from PBMC-derived monocytes

Isolated PBMCs from healthy adult donors were re-suspended in RPMI with 10% FBS. The re-suspended PBMCs were equally distributed onto 4-wells of a 24-well plate (different plates for different donors) and were incubated at 37^{o}C at 5% CO_2 for 2 hours. Subsequently, the supernatants containing non-adherent lymphocytes were discarded and the adherent monocytes were washed twice with PBS. After complete removal of PBS, RPMI with 10% FBS and 50 ng/ml human recombinant M-CSF (to aid monocyte differentiation to macrophages) was added to each well and the monocytes were incubated at 37^{o}C at 5% CO_2 for 6 days (without media change). Macrophage differentiation was confirmed via light microscopy and subsequently macrophages were used for cytokine treatments. (**Figure 4.1, section 4.4.1**)

2.2.1.2. THP-1 cell culture

The human monocytic THP-1 cell line has also been utilised to determine the effects of treatments of macrophages without the variation seen in human primary cells. THP-1 cells are non-adherent before differentiation is induced and were cultured in RPMI medium 1649 GlutaMAX supplemented with 10% FBS and 1% L-glutamine and 1% antimycotic/antibiotic in 75 cm^2 flasks which were placed vertically in a humidified chamber at 5% CO_2 at 37^oC. Upon confluence, the cells were counted and subsequently 750,000 cells per well were distributed into 6-well plates. 100 ng/ml PMA was added to each well for 4-5 days at 37^oC to promote attachment of the THP-1 cells to the bottom of the wells and differentiation towards macrophages (determined via light microscopy). Following completion of PMA incubation, PMA-containing media was discarded, and the THP-1 macrophages were incubated for 24 hours at 37°C at 5% CO_2 in PMA-free medium before being used for cytokine treatments and downstream experiments. (**Figure 4.2, section 4.4.1**)

2.2.2. Conditionally immortalised human GEnCs (ciGEnCs) culture

A cell line of human conditionally immortalised GEnCs (ciGEnCs) was developed and kindly donated by Professor Moin Saleem and Dr Simon Satchell (Academic Renal Unit, Bristol Royal Hospital for Children)[258]. Primary human GEnCs were obtained from healthy human glomeruli from donated children's kidney tissue, as previously done with human podocyte conditionally immortalised cell line development, which preceded the development of ciGEnCs[259]. The kidney tissue was transported and kept at all times prior to human glomeruli isolation at cool conditions[259].

For the development of conditionally immortalised podocytes, human glomeruli were isolated via differential sieving (using different sizes of sieves) up to 24 hours post-nephrectomy and sieved glomeruli were collected in a sterile container[259]. Subsequently, the

cortex which contains the glomeruli was isolated from the medulla, was chopped into small pieces using disposable scalpels and the cortex pieces were sieved again[259]. The isolated glomeruli were finally transferred onto culture-treated flasks or Petri dishes and were incubated at 37ºC[259]. After 7-10 days, the podocytes have sufficiently grown, and patches of podocytes are formed and reach confluence; at this stage the podocytes can be isolated from the cultured glomeruli via trypsinization and passing through a 40μM cell strainer[259].

The primary podocytes can then be plated into 25 or 75cm^2 flasks and they can now be transfected with vectors containing the temperature sensitive mutant of the Simian virus 40 large T-antigen (SV40LT) and the essential catalytic subunit of the human telomerase reverse-transcriptase (hTERT) gene for conditional immortalisation[318]. The SV40LT allowed for podocyte growth/proliferation at a permissive temperature (33ºC) in a non-differentiated state and subsequent maturation and differentiation after thermoswitching to a non-permissive temperature leading to SV40LT inactivation (37ºC)[319] whereas the hTERT gene aided prolonged ci podocyte culture, overcoming the common issue of early senescence of primary podocytes in culture[320]. To ensure that ci podocytes were indeed generated, podocyte-specific markers (such as nephrin and podocin) were tested and compared between primary and ci podocytes and no significant differences were observed[259].

Prior to conditional immortalisation, primary human GEnCs were isolated from donated human glomeruli. The glomeruli were isolated via differential sieving and subsequently were grown in cell culture treated flasks or Petri dishes[259]. Outgrowth of endothelial and epithelial cells (podocytes) was daily monitored and approximately after three to eight days, the endothelial cells were purified and detached from the epithelial cells via selective trypsinisation and further filtration via sieving, cells were pelleted and incubated with anti-PECAM-1 monoclonal antibody and finally glomerular endothelial cells were selectively isolated via

magnetic separation beads; the glomerular endothelial cells were then plated and cultured again[258,321]. The ciGEnCs were transfected with vectors for SV40LT and hTERT[258].

When thermo-switched to 37°C the ciGEnCs stop proliferating, terminally differentiate after approximately 5 to 7 days of culture and express at levels similar to primary human glomerular endothelial cells and in contrast to podocytes, endothelial cell-specific markers, such as PECAM-1, ICAM-2, VEGF receptor 2 (VEGFR2), and von Willebrand factor (vWF)[258]. This approach successfully overcomes the limited proliferative ability of primary GEnCs in culture and permits scientists to use ciGEnCs in various experiments, as it allows successful culture of ciGEnCs up to passage 40[258].

A similar approach has also been used for the generation of conditionally immortalised (ci) mesangial cells (MCs)[260]. After successful generation of the ci MCs, the mesangial cells were tested for expression of GEnC-specific and podocyte-specific markers and were found to be negative for these markers[260]. The ci MCs were able to express MC-specific markers (alpha-smooth muscle actin, PDGFR-β and fibronectin) and similarly to the primary MCs[260].

The ciGEnCs were cultured at 33ºC at 5% CO_2 in endothelial growth medium 2-microvascular containing 5% FBS and the following growth factors supplied together with FBS and the basal endothelial media; hydrocortisone, ascorbic acid, human endothelial growth factor (h-EGF), fibroblast growth factor (h-FGF) and insulin growth factor (h-IGF).VEGF was excluded as it was utilised for ciGEnC stimulation in certain experiments but also because the developers of the cell line excluded VEGF addition to the media so as to prevent ciGEnC hyperproliferation. The concentrations of the growth factors added in the media were not disclosed by the manufacturing companies for reasons of proprietary.

The ciGEnCs were grown in 25cm^2 culture flasks with media changed every 3-4 days as required. Upon confluence, the flasks were trypsinised with 0.25% trypsin for 10 minutes until

detached from the bottom of the flasks and at least a 3-fold higher amount of media compared to the total amount of trypsin was added in order to inactivate the trypsin. The ciGEnCs from each flask were separately added into 10 ml tubes, were pelleted by centrifuging at 200 xg for 5 minutes and were equally distributed in 6-well, 12-well or 24-well plates according to experimental needs and were incubated at 33^{o}C until 50% confluent. At that point, the plates were thermoswitched to 37^{o}C for 5-7 days until complete ciGEnC differentiation was achieved. Differentiation of ciGEnCs was confirmed via observing a change into a cobblestone, fibroblast-like morphology via light microscopy **(Figure 2.2)**. Subsequently, the ciGEnCs were treated at 37^{o}C for specific amounts of time with pro-inflammatory factors, sera or culture media from other cell types, according to experimental needs. At the end of the treatments, the conditioned ciGEnC culture media were collected and the ciGEnCs were used for downstream experiments.

For all experiments, ciGEnCs between passage 29 and 36 have been utilised with a minimum of 3 consecutive passages to ensure reproducibility and to allow for appropriate statistical analysis of the data. Untreated ciGEnCs cultured in medium without addition of any other factors were included in all experimental repeats to enable comparison of any treatment-induced changes with basal mRNA and protein production or ciGEnC viability levels.

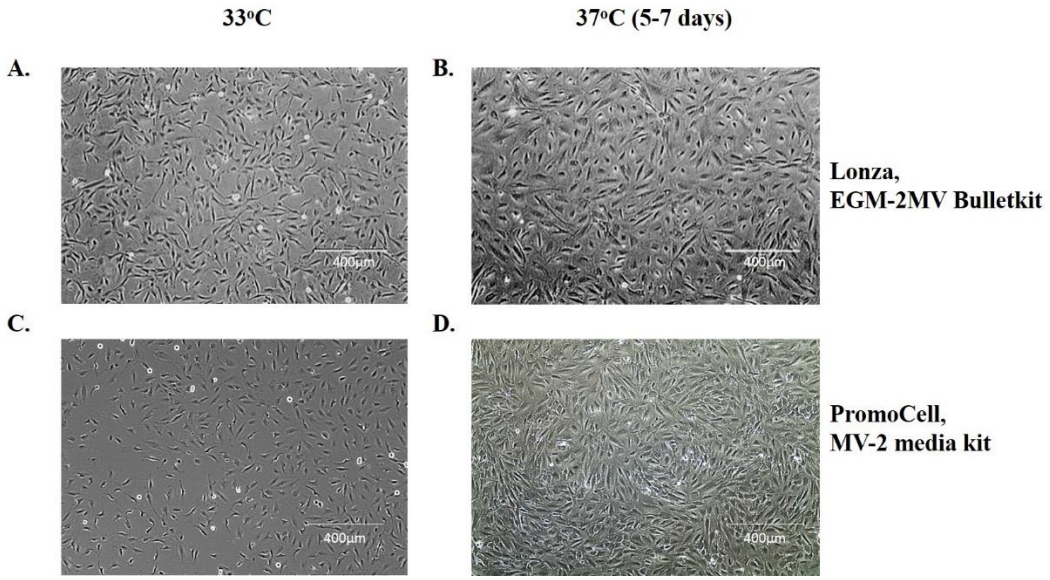


Figure 2.2 Cultured ciGEnCs at 33°C and 37°C.

At 33°C (A, C) ciGEnCs continuously proliferate. Once thermoswitched at 37°C for 5-7 days, proliferation ceases, the ciGEnCs differentiate and morphological changes (acquisition of a cobblestone, fibroblast-like morphology) can be confirmed by light microscopy. Similar morphology was seen between Lonza and PromoCell media. Scale bars: 400μm.

2.2.2.1.Serum treatments of ciGEnCs

The ciGEnCs were cultured in 25 cm^2 flasks and were seeded for 7 days at 37°C onto 12-well plates using the Lonza media supplemented with 5% FBS. Subsequently, the differentiated ciGEnCs were washed three times with PBS and were treated for 4 and 24 hours in Lonza medium free of FBS and supplemented instead with 5% serum from JSLE patients with active LN (renal BILAG; British Isles Lupus Activity Group: A or B, N=10), inactive LN (renal BILAG: D or E, N=10) as well as with 5% serum from age- and sex-matched healthy controls (N=10). Following incubation, the ciGEnCs were washed twice with PBS and RNA was collected for real-time PCR assays.

The BILAG index is an important tool that monitors disease activity via using the BILAG score. The BILAG score does not take into account immunological data but it consists of both

renal and haematological assessments, including; assessment of blood pressure, evaluation of proteinuria (urine dipstick, 24-hour quantification) and active urine sediment, presence of nephrotic syndrome, evaluation of renal function via creatinine/creatinine clearance measurement and GFR) and histological manifestation of active LN[322]. The renal BILAG score can effectively distinguish active from inactive LN and thus, is a very useful tool for accurate assessment of lupus renal disease activity[322].

Renal BILAG scores were previously assessed by experienced clinicians and were readily available for this study. However, although some factors included in the scoring (such as albumin:creatinine ratio) are quantitative values some are subject to inter- and intra-observer bias. For instance, one of the criteria is evidence of damage on renal biopsy and this requires the clinician to have requested a biopsy based on the evidence they have seen in the clinic; this level of evidence may differ based on the examining clinician (inter-observer bias) and it has to be noted that even when one specific clinician is examining a material more than once or on different days, a degree of variation may be observed (intra-observer bias). Furthermore, once a biopsy has been performed, it is analysed by histopathologists and a recent paper in childhood LN has demonstrated that the inter-observer variability is significant[323]. In an attempt to minimise bias, I selected active LN samples based, along with the BILAG score, on records of biopsy-proven lupus nephritis where the biopsy had taken place within 3 months of sample acquisition.

2.2.2.2.Cytokine and LPS treatments with ciGEnCs

Differentiated ciGEnCs were seeded in 6-well or 12-well plates for flow cytometry assays, in 6-well plates for WB experiments, in 12-well plates for SYBR Green real-time PCR and ELISA assays and in 24-well plates for mRNA dose response SYBR Green real-time PCR assays. Subsequently, the cells were incubated at 37 ºC for 15 minutes, 30 minutes, 4 hours, 24

hours and 4 days with 10 ng/ml of seven human recombinant cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-13, VEGF) either individually or in a combined cytokine treatment and with 1 μg/ml of LPS. A 24-hour dose response experiment for the cytokines found to have a robust effect on ciGEnCs *in vitro* activation (TNF-α, IL-1β, IFN-γ and IL-13) was performed using 10-fold serial cytokine dilutions (10 pg/ml – 10 ng/ml).

2.2.2.3.Urinary biomarker treatments

The effect of six previously determined novel urinary biomarkers for LN (AGP, CP, TF, L-PGDS, MCP-1 and NGAL) [324–326] on the ciGEnCs was tested after either individual biomarker treatment or combined treatment of all six biomarkers together. The biomarkers were added in high and low concentrations, reflecting their concentrations as detected in the urine of patients with active LN (high concentration) or inactive LN (low concentration)[327], as specified in **section 5.5.1.1**.

2.2.3. SYBR-Green real-time PCR principles

Polymerase Chain Reaction (PCR) is a technique utilised to create multiple copies of a target DNA sequence (in this case, complementary DNA (cDNA) derived from reverse transcription (RT) of messenger RNA (mRNA)).[328] Amplification of the double-stranded target DNA occurs with the use of a solution containing a DNA polymerase enzyme, a mix of all four nucleotides and a pair of single-stranded primers (forward and reverse) complementary to the target DNA sequence. When mixed with the DNA polymerase and nucleotide solution, the target DNA is amplified as follows: initially the mixture is heated up to 95^{o}C to induce denaturation of the double-stranded DNA (ds-DNA) and subsequently the temperature is reduced to 55^{o}C to promote annealing of the primers to the target DNA strands. Following the annealing step, the temperature is increased to 75^{o}C to promote extension of the DNA sequence

of interest and amplification of the specific DNA target through repeated cycles of the annealing and extension steps.

The SYBR-Green method can be used for real-time PCR analysis of the amplified DNA target. [329] It is based on the ability of the SYBR-Green dye to preferentially bind to the minor groove of ds-DNA, leading to formation of a DNA - SYBR-Green complex which absorbs blue light and emits green light **(Figure 2.3)**. The more ds-DNA products accumulate over time, the more the SYBR-Green dye fluorescence signal will increase and become more intense. Therefore, the more highly expressed the DNA target is, the higher levels of fluorescence will accumulate over time, and vice versa.

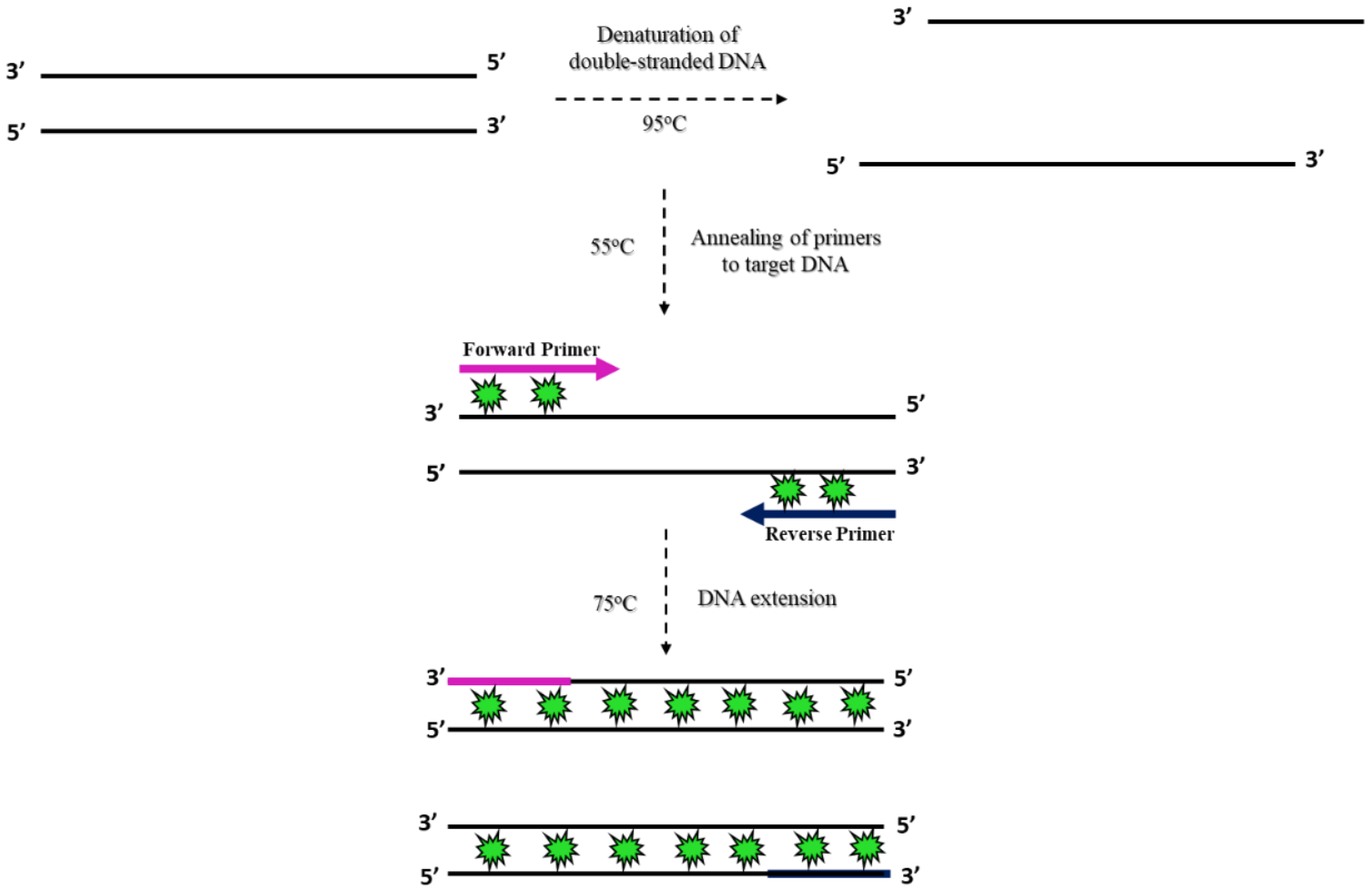


Figure 2.3 SYBR Green real-time PCR.

Forward and reverse primers bind the target DNA sequence and through repeated cycles of the annealing and extension steps multiple copies of the target DNA are produced. SYBR Green dye is incorporated into the double-stranded DNA.

2.2.3.1.RNA extraction

Following completion of treatment periods according to the requirements of each experimental set-up, the ciGEnCs in cell culture plates were washed twice in PBS and 1 ml of Trizol reagent was added into each well for a minimum of 10 minutes at room temperature. The lysed cells were then briefly scraped from the bottom of the wells and the Trizol cell lysate was transferred into 1.5 ml tubes. 200 µl of chloroform was added into each of the tubes and shaken by hand to mix for 15s. Following this the tubes were left at room temperature to settle for 2-3 mins and then centrifuged at 12,000 xg for 15 minutes at 4^{o}C to aid separation of RNA from DNA and protein. Subsequently, the upper aqueous phase containing RNA was removed (leaving the lower organic phase with DNA and proteins) and added into new 1.5 ml tubes, to this 500 µl isopropanol was added. At this point, the isopropanol-precipitated RNA was frozen at -80^{o}C for future utilisation or was left to precipitate for a minimum of 10 minutes at room temperature and subsequently samples were centrifuged at 12,000 xg for 30 minutes at 4^{o}C. The pelleted RNA was then re-suspended in 500 µl of 70% ethanol and was centrifuged at 12,000 xg for 15 minutes at 4^{o}C to aid further precipitation and concentration of the RNA. The ethanol was removed, and the samples were left to air-dry for 1 minute at room temperature before re-suspension into 100 µl of nuclease-free water followed by addition of 250 µl of absolute ethanol (100%).

Subsequently, RNA on-column clean-up and on-column DNase digestion were performed using the Qiagen RNeasy Mini Kit as follows; 350 µl of Buffer RLT (lysis buffer) were added into each nuclease-free water and ethanol- diluted sample and samples were thoroughly mixed to ensure complete lysis of any remaining cell fragments. The mixture (approximately 750 µl) was then transferred into columns placed on supplied collection tubes. The columns were centrifuged at 8,000 xg for 15 seconds at room temperature and placed into new collection

tubes. Each column was then washed with 350 µl RW1 wash buffer, the columns were centrifuged at 8,000 xg for 15 seconds and after emptying the collection tubes DNase digestion was performed by adding a mixture of 80 µl of RNase-free DNase diluted 8x into RDD buffer (RNase-free DNase set - Qiagen) directly onto the membrane of each column. The columns were then incubated with the DNase/RDD mix for 15 minutes at room temperature and at the end of this incubation 350 µl of RW1 buffer were added into each column followed by a 15-second centrifugation at 8,000 xg. Following thorough emptying of the collection tubes, 500 µl of buffer RPE (supplied with ethanol to aid washing of membrane-bound RNA and ensure removal of residual salts) were added into each column and columns were centrifuged at 8,000 xg for 2 minutes. This process was repeated twice. Following completion of RPE washes, the columns were placed into clean and sterile 1.5 ml tubes and purified RNA was eluted by adding 40 µl of nuclease-free water into each column and centrifuging at 8,000 xg for 2 minutes.

The extracted RNAs' concentrations as well as 260/280 (RNA purity ratio, indicating potential presence of protein, phenol or other contaminants that strongly absorb at 280 nm) and 260/230 (RNA purity ratio, indicating potential presence of contaminants such as phenol and guanidine that absorb at 230 nm) ratios were determined using Nanodrop (ND-1000 Spectrophotometer, Thermo Fisher Scientific).

2.2.3.2. cDNA conversion and real-time PCR for relative quantification of mRNA expression

Quantitative SYBR Green real-time PCR was used to assess mRNA levels of different genes expressed by the ciGEnCs.

2.2.3.3. Agilent kit used for reverse transcription and real-time PCR

RT was initiated from 100ng or 200ng of total RNA diluted in nuclease-free water to equal concentration per experiment using 15ng/ml of random primers (initial concentration: 100

ng/ml) per sample. The samples were vortexed briefly, centrifuged and incubated at 65^{o}C for 5 minutes to enable denaturation of the RNA secondary structures.

Subsequently, the mastermix was prepared as per manufacturer's instructions: 1X affinityScript RT buffer (initial concentration: 10x), 4mM of deoxy-ribonucleotides (dNTP) mix (initial concentration: 100mM, 25mM each), 1U/µl of RNAse inhibitor (initial concentration: 40U/µl) and 1 µl of affinityScript reverse transcriptase (RTase). The final volume of each reaction was 20 µl. Three RNA samples were randomly chosen as negative controls and RTase was replaced with nuclease-free water for these reactions. All samples were thoroughly mixed and centrifuged and were incubated in the thermal cycler using the following programme: 25°C for 10 minutes- 42°C for 60 minutes-72°C for 15 minutes.

For the real-time SYBR Green PCR, all cDNAs and primers were diluted 1:10 in nuclease-free water. The Mastermix (Brilliant SYBR-Agilent Kit) for each sample was prepared as per manufacturer's instructions: 0.2µl of sterile H_2O, 5µl of SYBR Green qPCR mastermix, 0.3µl of ROX dye (reference dye), and 0.25µl of 10x diluted forward and reverse primers were thoroughly mixed, vortexed and centrifuged.

Following Mastermix preparation, 6µL of Mastermix and 4µL of diluted cDNA were loaded in duplicates onto 96-well plates (final volume: 10 µl per well). For the negative control samples, the cDNA was replaced with 4µl of sterile H_2O. All samples were thoroughly mixed and centrifuged before qPCR runs.

Real-time PCR took place using Stratagene MX thermal cycler and MxPro software under the following conditions: 95°C for 3 minutes (initial denaturation) followed by 40 cycles at 95°C for 20 seconds (denaturation) and then 60°C for 20 seconds (annealing/extension) with fluorescence reading after each cycle. For the generation of the dissociation curve, the

temperature was increased to 95°C for 1 minute followed by a decrease to 55°C and finally the temperature was ramped up to 95°C (0.2°C/cycle) while constant fluorescence was collected.

2.2.3.4. Primerdesign kit used for reverse transcription and real-time PCR

RT was initiated from 100ng or 200ng of RNA. RNA was added onto PCR tubes previously loaded with 20 μl RT premix (Precision nanoScript™ 2 RT kit). All samples were vortexed briefly and were spun down and subsequently they were incubated in the thermal cycler using the following programme: 42°C for 20 minutes (RT step) and 72°C for 10 minutes (heat inactivation for reverse transcriptase).

The cDNA was subsequently diluted using nuclease-free water and real-time PCR reactions (which took place onto 96-well plates) were prepared as follows: 1x Precision®PLUS qPCR Master Mix (initial concentration: 2x) were mixed with 6 pmols of forward and 6 pmols of reverse primers and with nuclease-free water up to a volume of 7 μl per well (a premix was prepared according to the total number of cDNA samples which were loaded on the 96-well plates in duplicates). Subsequently, 3 μl of diluted cDNA were added into each well reaching a total volume of 10 μl per well.

Real-time PCR took place using Stratagene MX3000P thermal cycler and MxPro software under the following conditions: 95°C for 2 minutes (initial denaturation) followed by 40 cycles at 95°C for 10 seconds (denaturation) and then 60°C for 1 minute (annealing/extension) with fluorescence reading after each cycle. For the generation of the dissociation curve, the temperature was increased to 95°C for 1 minute followed by a decrease to 55°C and finally the temperature was ramped up to 95°C (0.2°C/cycle) while constant fluorescence was collected.

2.2.3.5.Relative quantification of transcripts

Relative quantification of transcripts was determined using Delta Ct (cycle threshold) values (ΔCt). mRNA expression for each gene was normalised to one or more housekeeping (HK) genes by calculating the ΔΔCt values. For the calculation of the ΔΔCt values, the mean (for one HK) or the geometric mean (for more than one HKs) of the most suitable of the following HK gene(s) was used: beta-actin (ACTB), TATA-binding protein (TBP), beta-tubulin (TUBB) and 14-3-3 protein zeta/delta (YWHAZ). Suitability of the HK gene was determined each time by comparing all the treatment groups of each experiment to each other, using the same non-parametric statistical analysis that was eventually utilised for statistical analysis of the target genes. Only the HK gene(s) having the most similar expression levels among groups without displaying any statistically significant differences in mRNA expression were used for each experiment. For some experiments, only one HK gene could meet these criteria and thus, for these cases, target genes were compared to the mean of this HK gene instead of the optimal comparison to the geometric mean of at least three HK genes.

The equation utilised for the ΔΔCt values is the following:

$$\Delta\Delta Ct = 2^{-(\text{Mean } \mathbf{[target\ gene]} - (\text{Geo})\text{Mean } \mathbf{[HK\ gene(s)]})}$$

2.2.3.6.Design of primer pairs and calculation of efficiencies

Initially, through PubMed's "Gene" search categories, the "mRNA-protein" accession number of the human genes of interest was collected and was used to design the primer pairs of the target genes through the Primer-BLAST tool. The following options were chosen for optimal design of the forward-reverse primer pairs; PCR product size had to be between 85-200 base pairs (bp), the G-C content of primers and melting temperature (Tm) had to be 50-60% and 50ºC-65ºC respectively, whereas the primers should preferentially cross an exon-

exon junction. Furthermore, targets with 2 or more mismatches were ignored and primers with four or more repeats of the same nucleotide were avoided. Quality scores and primer dimer formation of all the potential primer pairs generated from Primer-BLAST were determined using the Amplifx software. Only primers that did not form any dimers and with a quality score higher than 70 and generally marked as "Good" were purchased.

ACTB primers were purchased from Invitrogen whereas the rest of the primers used for SYBR-Green real-time PCRs were purchased from Eurofins (100 μM). Primers are illustrated on **Table 2.7.** Primer efficiencies were calculated by using 1:10 diluted cDNA (neat cDNA) from 3 random ciGEnCs samples as a stock and by performing serial dilutions (1:2, 1:5, 1:7 and 1:10). All samples of neat and serially diluted cDNA were loaded onto 96-well PCR plates as triplicates and real-time PCRs for all candidate primer pairs were performed. Results were analysed via linear regression using the GraphPad Prism 4.0 software and the slope of the linear regression curve was used to calculate the primer efficiencies using the Thermo Fisher Scientific software. Only pairs of primers with efficiencies within the range 80%-120% were utilised for experiments.

Gene	Forward Primer Sequence (5'- 3')	Reverse Primer Sequence (5'- 3')
ACTB	CATTGCGGTGGACGATGGA	AGATCAAGATCATTGCTCCTCCTG
TBP	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
TUBB	GGACCGCATCTCTGTGTACT	CTGCCCCAGACTGACCAAATA
YWHAZ	ACTGGGTCTGGCCCTTAACT	GGGTATCCGATGTCCACAATGTC
TNF-α	CTTCTGCCTGCTGCACTTTG	GGGTTTGCTACAACATGGGC
IL-1β	CAACAGGCTGCTCTGGGATT	CCTGGAAGGAGCACTTCATCT
IL-6	AGAGGCACTGGCAGAAAACA	TCACCAGGCAAGTCTCCTCA
IL-8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA
IL-10	GCGCTGTCATCGATTTCTTCC	GCCACCCTGATGTCTCAGTT
MIP-1α	CATTCCGTCACCTGCTCAGA	AGCAGCAAGTGATGCAGAGA
IP-10	AGCCCCACGTTTTCTGAGAC	GAGAGAGGTACTCCTTGAATGCC
M-CSF	CCCCAGTCCTCTCTTAAAAGGC	GAGAGGACCCAGGCAAACTT
GM-CSF	GAGACACTGCTGCTGAGATGAA	AGGAAGTTTCCGGGGTTGGAG
MCP-1	CTCGCTCAGCCAGATGCAAT	TCTCCTTGGCCACAATGGTC
VCAM-1	GGTGGGACACAAATAAGGGT	GCTTGAGAAGCTGCAAAC
TF	TTCACTGGCCCAAGTGGTTT	AGAAGGAAGGGCTCAGTTGC
L-PGDS	CCAGGGCTGAGTTAAAGGAGA	CCCTGGGGAGTCCTATTGTTC
TNFR1	CTGGAGCTGTTGGTGGGAAT	TTGTGGCACTTGGTACAGCA
TNFR2	CACCGGGAGCTCAGATTCTTC	CACTGTGAGCTGTGGTCAGA
IL-1RI	GCGGCAGGAATGTGACAA	TACCAAGGGGTCCAGCTTCT
IL-1RII	TGAGCAGCAACAAGGCCA	CAACACGTACAAGCGCAACA
COL1A1	CCACGCATGAGCGGACCCTAA	ATTGGTGGGATGTCTTCGTCTTGG
COL1A2	ACAAGGCATTCGTGGCGATA	ACCATGGTGACCAGCGATAC
COL3A1	GACCTGGAGAGCGAGGATTG	GTCCATCGAAGCCTCTGTGT
COL4A1	GCCAGCAAGGTGTTACAGGATT	AGAAGGACACTGTGGGTCATCTATT
LAMB1	CCGGAAAGGAAGACGGGAAG	CGCCAGGTCCTGCTGTTTCTAA
LAMB2	CAGGCAGAGTTGACACGGAA	AGCCAGCACGCTTAGCAGTAG
TIMP1	GGAATGCACAGTGTTTCCCT	GCCCTTTTCAGAGCCTTGGA
MMP2	CCATGAAGCCCTGTTCACCA	CTTCTTGTCGCGGTCGTAGT
MMP7	GGGAACAGGCTCAGGACTAT	CTCCACATCTGGGCTTCTGC
MMP9	GGCGCTCATGTACCCTATGT	TTCAGGGCGAGGACCATAGA
FN	TCGCAGCTTCGAGATCAGTG	CCCCTCTTCATGACGCTTGT

Table 2.7 List of primers used for real-time PCR experiments.

2.2.4. Flow cytometry (FC)

2.2.4.1. Basic principles of FC

FC allows for the measurement of optical features and fluorescence characteristics of single cell suspensions [330]. Different cell populations can be distinguished with FC according to their size (illustrated by the forward scatter or FSC) and granularity/internal complexity (illustrated by the side scatter or SSC). **(Figure 2.4)**

Depending on the aims of the assay, cells can be labelled with DNA- or RNA-binding/intercalating dyes or with fluorochrome-conjugated antibodies against target proteins, either membrane–bound or intracellular. Propidium iodide (PI) is an example of a commonly used DNA-intercalating dye, which cannot penetrate the cell membrane and is therefore very useful in distinguishing between viable, early and late apoptotic cells. Furthermore, among fluorochromes commonly utilised for Ab conjugation are Fluorescein isothiocyanate (FITC), allophycocyanin (APC), phycoerythrin (PE) and Alexa Fluor 488 (A488). Multiple staining of the same cell sample with antibodies against more than one target protein conjugated with fluorochromes emitting light of non-overlapping or partially overlapping wavelengths, can aid simultaneous detection of changes in the levels of these target proteins.

Once inside the flow cytometer, the suspended cells are focused into a laminar stream flow generated by an isotonic sheath fluid which allows each cell to pass separately through an "inspection point" (Fluidic System, **Figure 2.4)**. The Merck Guava flow cytometer that was specifically used for this thesis'experiments however, uses a patented microcapillary system without sheath fluid to create a single cell suspension to allow each cell to pass separately through an "inspection point". There, a monochromatic light beam of a certain wavelength – usually from a laser source- excites the fluorochromes to a state of higher energy. Upon return to their normal energy state, the excited fluorochromes will emit light of a higher wavelength than that of the monochromatic laser light beam. Light that passes straight through the cell

(FSC) and light that is scattered 90° (SSC) are collected with the help of an optical system consisting of filters allowing for only certain wavelengths to be directed forward (filters can also act as dichroic mirrors able to disperse light at a 90^{o} angle) and different light detectors able to capture light emitted from fluorochromes or fluorescent dyes (FL1, FL2, FL3) **(Figure 2.4)**.

The light signals are finally amplified by photomultiplier tubes and are digitalised to allow for data analysis and visualisation using a computer **(**Electronic System, **Figure 2.4).**

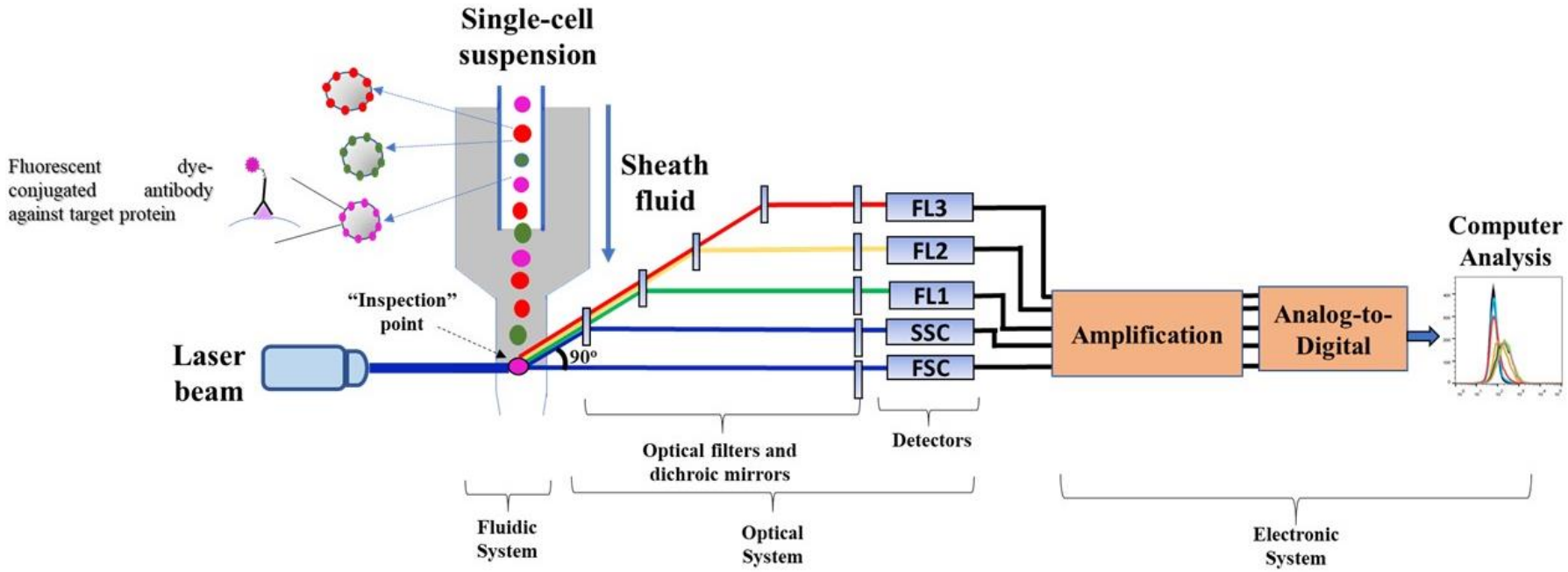


Figure 2.4 Flow Cytometry.

A flow cytometer consists of three interconnected systems; the microcapillary system (which focuses cells into a single stream), the optical system (consisting of a monochromatic laser beam which "interogates" each cell separately, a series of optical filters and dichroic mirrors and FS, SS and fluorescence detectors) and the electronic system which leads to amplification of light signals emitted by the detectors and conversion to digital signal for visualisation via computer analysis.

2.2.4.2.Basic principles of apoptosis assay using FC

Apoptosis is a type of genetically programmed cell death characterised by successive and distinct morphological alterations eventually leading to loss of cell viability[331]. As apoptosis is a tightly regulated process and each event leading to gradual cell fragmentation occurs in a specific order, changes in plasma cell membrane polarity take place early in the apoptotic procedure and precede changes in membrane integrity[332,333]. Therefore, early and late apoptotic events such as phosphatidylserine (PS) translocation from the inner to the outer surface of the cell membrane and increased membrane permeability to otherwise non-permeable DNA-intercalating dyes respectively, can be utilised to distinguish among different apoptotic stages. [332,333] Annexin V is a protein able to bind phospholipids such as PS and once conjugated to fluorochromes such as FITC, can aid sensitive detection of cells at early stages of apoptosis. [333] Furthermore, PI is not able to penetrate the plasma membranes of viable cells but once the membranes lose their integrity during later apoptotic stages, PI can enter the cells and bind their DNA[333]. Therefore, a combination of Annexin V – FITC and PI staining can help distinguish over time between viable (Annexin V/PI negatives), early (Annexin V positives/PI negatives) and late apoptotic cells (Annexin V/PI positives) **(Figure 2.5)**.

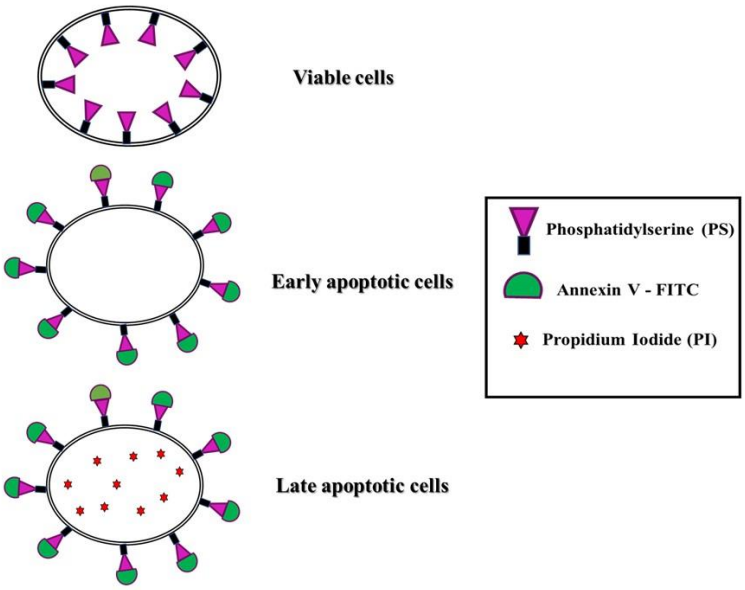


Figure 2.5 Annexin V- FITC and PI staining for distinction between viable, early and late apoptotic cells.

As PS is found on the inner side of the membrane of viable cells, annexin can bind phosphatidylserine only in early and late apoptotic cells. Furthermore, only late apoptotic cells are PI-positive. Therefore Annexin/PI staining can distinguish among viable, early and late apoptotic cells.

2.2.4.3. Fluorescence FC

For the FC assay, the ciGEnCs were treated for 4 and 24 hours with 10 ng/ml of TNF-α, IL-1β, IL-1 and, IFN-γ and 1μg/mL LPS. Subsequently, the effect of these cytokines and of LPS were investigated regarding potential changes on surface expression of ICAM-1, Vascular Cell Adhesion Molecule 1 (VCAM-1), E-/P-selectin, Programme Death-Ligand 1 (PD-L1) and Inducible Costimulator-Ligand (ICOS-L). Isotype controls were each time added at the same concentration as the antibodies against the target proteins.

At the end of 4- and 24-hour treatments, the ciGEnCs were washed twice with warm PBS and were subsequently trypsinised by adding 0.25% trypsin for 10 minutes at 37ºC. The trypsinised ciGEnCs were centrifuged at 300 xg for 5 minutes. Sample labelling took place using 96-well round bottom well plates. Each sample of ciGEnC treatments was re-suspended

in 50 μl of PBS with 0.1% BSA and was then transferred into a well of the 96-well plate. Subsequently, the antibodies were added in appropriate dilutions and the samples were incubated on ice and in the dark for 30 minutes to prevent photobleaching. Double or triple Ab staining was performed based on the wavelength of the light emitted from each fluorochrome upon excitation. After the 30-minute incubation the 96-well plates were centrifuged at 300xg for 30 seconds, supernatants were discarded, and the samples were washed by adding 200 μl of PBS-0.1% BSA in each well. The samples were subsequently centrifuged at 300xg for 30 seconds and a total of three similar washes were performed. Finally, the samples were re-suspended in 200 μl of PBS-0.1% BSA, were transferred into new 1.5 ml tubes and were directly run through the flow cytometer (Guava, Merck). Acquisition of geometric mean fluorescence intensity values for the fluorescence FC adhesion molecule and cytokine receptor assays and diagrams were generated using the FlowJo 10.3 Software.

2.2.4.4. Apoptosis Assay with FC

For the apoptosis assay, following 24-hour TNF-α, IL-1β, IL-13, IFN-γ and LPS treatments, the ciGEnC samples were trypsinised, centrifuged at 300 xg and resuspended in 100 μl HBSS and were transferred onto round bottom 96-well plates. The samples were stained with Annexin V-FITC at a 1:100 dilution on ice/dark for 15 minutes and subsequently, with PI at a 1:1000 dilution in dark and room temperature for 3 minutes. Following completion of Annexin V/PI incubation, the cells were further resuspended into 100 μl HBSS, were transferred back into 1.5 tubes and were directly run through the flow cytometer. Dot plots for the apoptosis assays were generated using the FlowJo 10.3 Software.

2.2.5. ELISA

2.2.5.1.Basic principles of sandwich ELISA

A sandwich ELISA is a technique utilised for detection and quantitative measurement of the concentration of a target protein in any sample containing proteins (such as cell lysates, supernatants, conditioned media, blood serum or plasma samples etc.) without requiring previous protein purification[334]. The target protein is quantified as it is sequestered between two different layers of antibodies, the capture and the detection Ab which are both specific to the target protein but bind different epitopes of the protein. The capture Ab is diluted in PBS and added overnight to coat the bottom of the wells. Subsequently, a solution containing a blocking protein such as bovine serum albumin (BSA) is added to prevent non-specific binding. The sample is loaded onto the plate and incubated for 2h at room temperature for binding to occur. Following this the plate is washed in PBS + 0.05% Tween-20 and this step is followed by the addition of the detection Ab which is conjugated to streptavidin, with horseradish peroxidase (HRP) being used as a streptavidin substrate. This leads to the target protein being "sandwiched" between the capture and the detection Ab. Subsequently, addition of a chromogenic substrate for HRP such as Tetramethylbenzidine (TMB), causes a concentration-dependent colour change that can be quantified by comparison to a standard curve of known concentrations. Once the reaction has finished a stop solution is added (5% sulfuric acid diluted in ddH_2O) and the reaction is immediately measured at 450nm using a microplate reader (**Figure 2.6**). For the measurement of the unknown concentrations of the target protein in different samples, a standard sample of known concentration is serially diluted and added onto the 96-well plate to create a standard curve (**Figure 2.7**). The equation of the standard curve (the R-square value of the standard curve should ideally range between 0.9-1.) allows for calculation of the unknown concentration of the target protein within different samples. All standards and samples are added onto the plates in duplicates to ensure better reproducibility

of the experiments whereas blank samples (consisting only of reagent diluent) are added as negative controls.

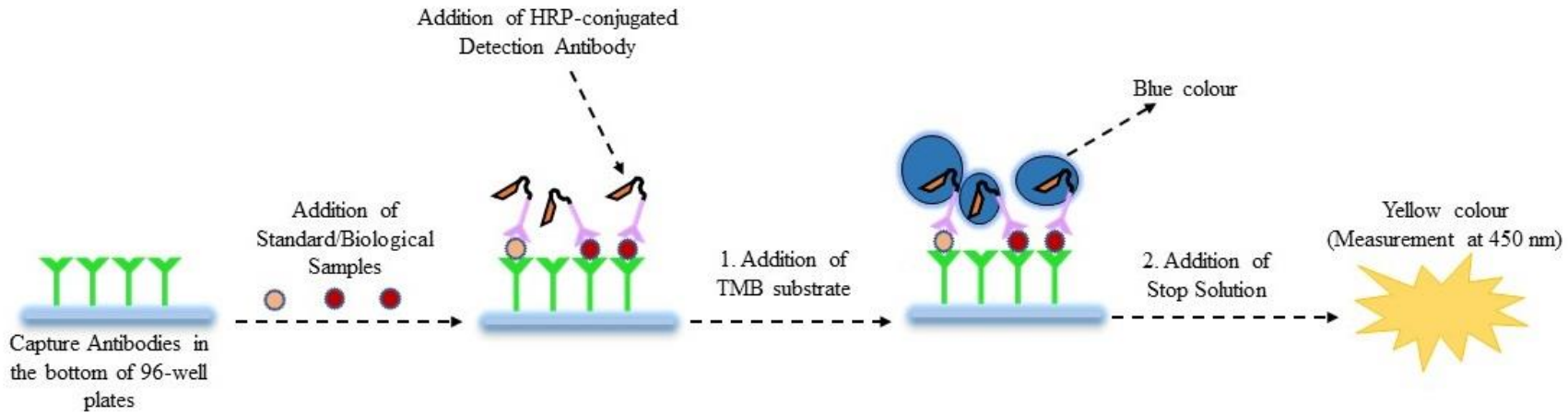


Figure 2.6 Sandwich ELISA.

A capture Ab is added on the bottom of the wells of the 96-well plates followed by addition of standards and samples and then addition of a detectionAb, often conjugated to an enzyme, such as HRP. Supplementation of chromogenic enzyme substrate such as TMB can lead to a chromogenic reaction and colour formation (HRP-TMB reaction: blue colour). Subsequent addition of a stop solution can lead to change of blue to yellow colour which can be easily processed and analysed at 450 nm via an Optical Plate Reader.

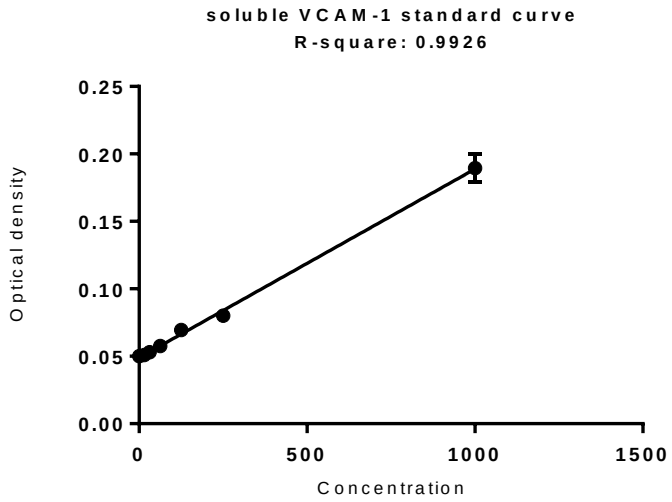


Figure 2.7 Example of a sandwich ELISA standard curve.

2.2.5.2.Protocol for sandwich ELISA

The secretion of MCP-1 and sVCAM-1 by ciGEnCs and secretion of TNF-α and IL-1β by THP-1 cells was assessed using single ELISA kits. All steps were followed as per manufacturer's instructions and as presented on this thesis's "Basic principles of sandwich ELISA" (**section 2.5.1**). A 2-fold dilution of all samples was performed before addition on 96-well plate. Plates were read directly at 450 nm using a Microplate Reader (Spectrophotometer).

2.2.5.3.Luminex multiplex ELISA principles

Unlike conventional ELISAs, a Luminex ELISA allows for simultaneous detection of multiple analytes of interest within the same sample.

Colour-coded magnetic microparticles pre-coated with analyte-specific capture antibodies are pipetted onto the 96-well plate of the Magnetic Luminex® Assay multiplex kit **(Figure 2.8)**. Subsequently, Luminex assay standards and samples are added onto the plates and the capture antibodies specifically bind the multiple analytes of interest **(Figure 2.8)**. Following

thorough washes for removal of non-bound substances, a biotin-conjugated Ab cocktail specific to the target analytes is pipetted into the wells of the plate and the plate is washed again (for the washes the plate is placed on top of the magnet to prevent microparticle loss) **(Figure 2.8)**. Finally, Streptavidin conjugated to PE is added onto each well and as streptavidin binds biotin, PE fluorescence can lead to a light emission where intensity is proportional to the concentration of the analytes of interest within each sample **(Figure 2.8)**. After a final wash following Streptavidin-PE addition, a buffer is added onto each well to re-suspend the microparticles and the plate is read using the Luminex® MAGPIX Analyzer. The Analyzer contains a magnet which focuses the microparticles onto a monolayer and a dual laser dissects the microparticles, utilising two Light Emitting Diodes (LEDs) which display differential spectrum patterns **(Figure 2.8)**. The two LEDs have distinct roles which are identification of the detected analyte and determination of the intensity of PE-signal **(Figure 2.8)**. Each well of the 96-well plate is scanned with a CCD camera. For this thesis' experiments, the Luminex® MAGPIX CCD Imager has been utilised.

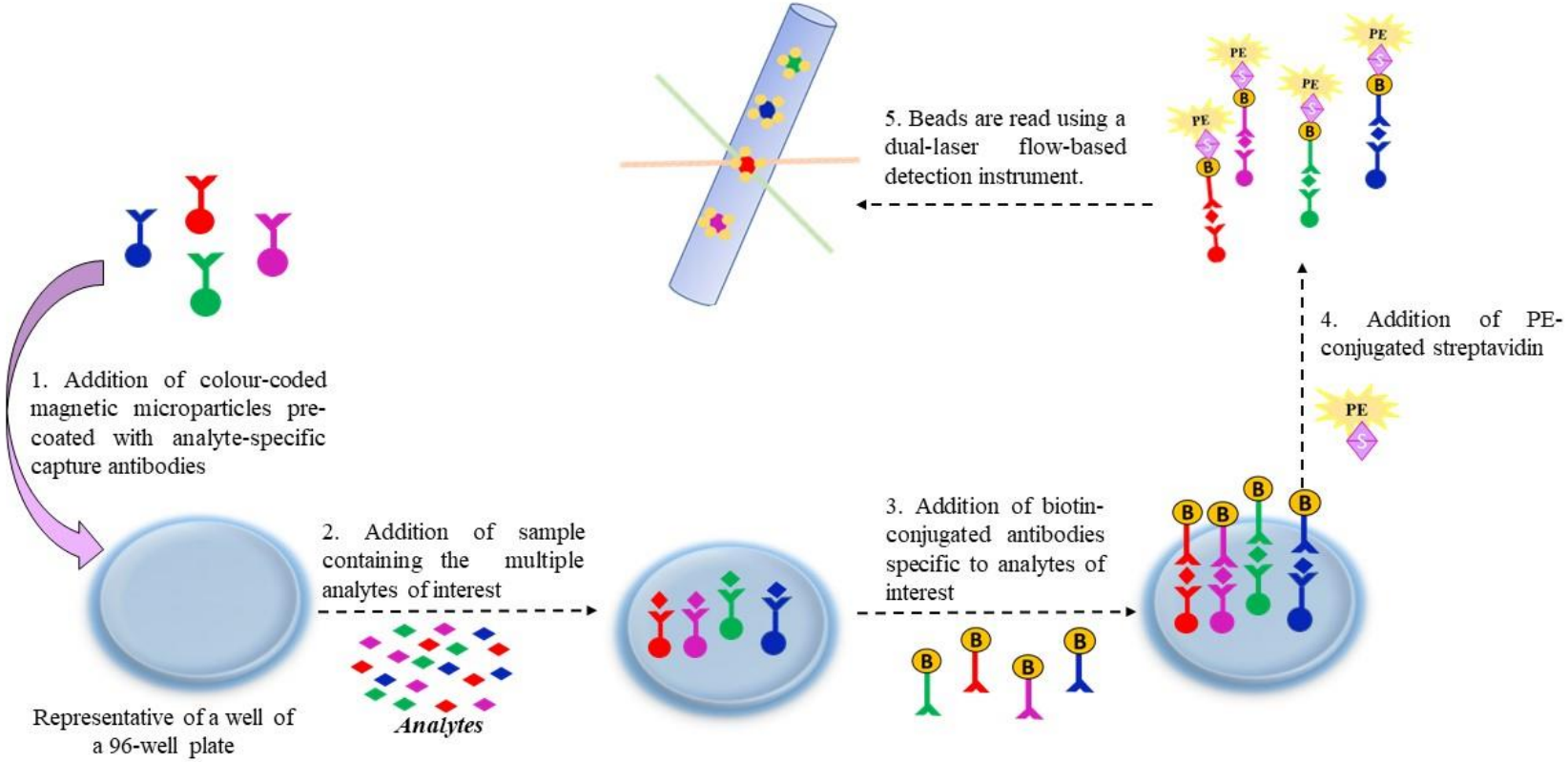


Figure 2.8 Luminex multiplex ELISA.

A Luminex ELISA allows for simultaneous detection of more than one analyte within the same sample. This technique utilises colour-coded magnetic microparticles which are pre-coated with analyte-specific capture antibodies. The microparticles are pipetted first on the wells of the 96-well plates and then the standards and samples are added followed by analytes-specific biotin-conjugated antibodies and subsequent addition of streptavidin-conjugated PE. The beads are then measured through a dual-laser flow-based instrument.

2.2.5.4.Protocol for Luminex multiplex ELISA assay

MIP-1α, IP-10, IL-6, IL-8, GM-CSF, M-CSF, TNF-α and IFN-γ secretion by the ciGEnCs was assessed via Luminex multiplex ELISA. All steps were followed as per manufacturer's instructions and as presented on this thesis's "Luminex multiplex ELISA principles" (**section 2.5.3**). A 2-fold dilution of all samples was performed before addition on 96-well plate. Each plate was analysed within 20 minutes using the Luminex® MAGPIX CCD Imager.

2.2.6. Western blotting

2.2.6.1. Basic principles of WB

WB is a semi-quantitative analysis technique allowing for Ab-based detection of a target protein within a mixture of various proteins[335].

In WB, biological samples of extracted proteins are initially loaded onto a polyacrylamide gel. The proteins are then separated according to their molecular weight (MW) via gel electrophoresis, with the proteins of higher MWs moving more slowly from the top to the bottom of the gel compared to those of lower MWs, when the electrical current is stably applied to the gel. A WB protein ladder consisting of recombinant proteins of a known range of MWs is always loaded onto the gel to aid accurate identification of the target protein. At the end of the electrophoresis, the separated proteins are transferred onto a PVDF membrane and the membrane is blocked for a specific amount of time to avoid non-specific binding of antibodies to its surface **(Figure 2.9)**.

Following blocking, a primary Ab specific for the target protein is added and binds the target protein on the surface of the membrane, Subsequently, a secondary Ab conjugated to an enzyme such as HRP is also added to specifically bind the primary Ab (the secondary Ab is specific to the host species of the primary Ab). When the appropriate substrate is provided for

the enzyme such as luminol for HRP, then HRP with the help of hydrogen peroxide (H_2O_2) will catalyse the oxidation of luminol, a process resulting into light emission (known as enhanced chemiluminescence or ECL) [336] **(Figure 2.9)**.

The emitted light is then detected, and the results of the WB chemiluminescent signal can be captured -among other ways- utilising digital imaging methods and instruments, leading to visualisation of the protein bands. Higher band intensity indicates higher protein expression and vice versa **(Figure 2.9)**.

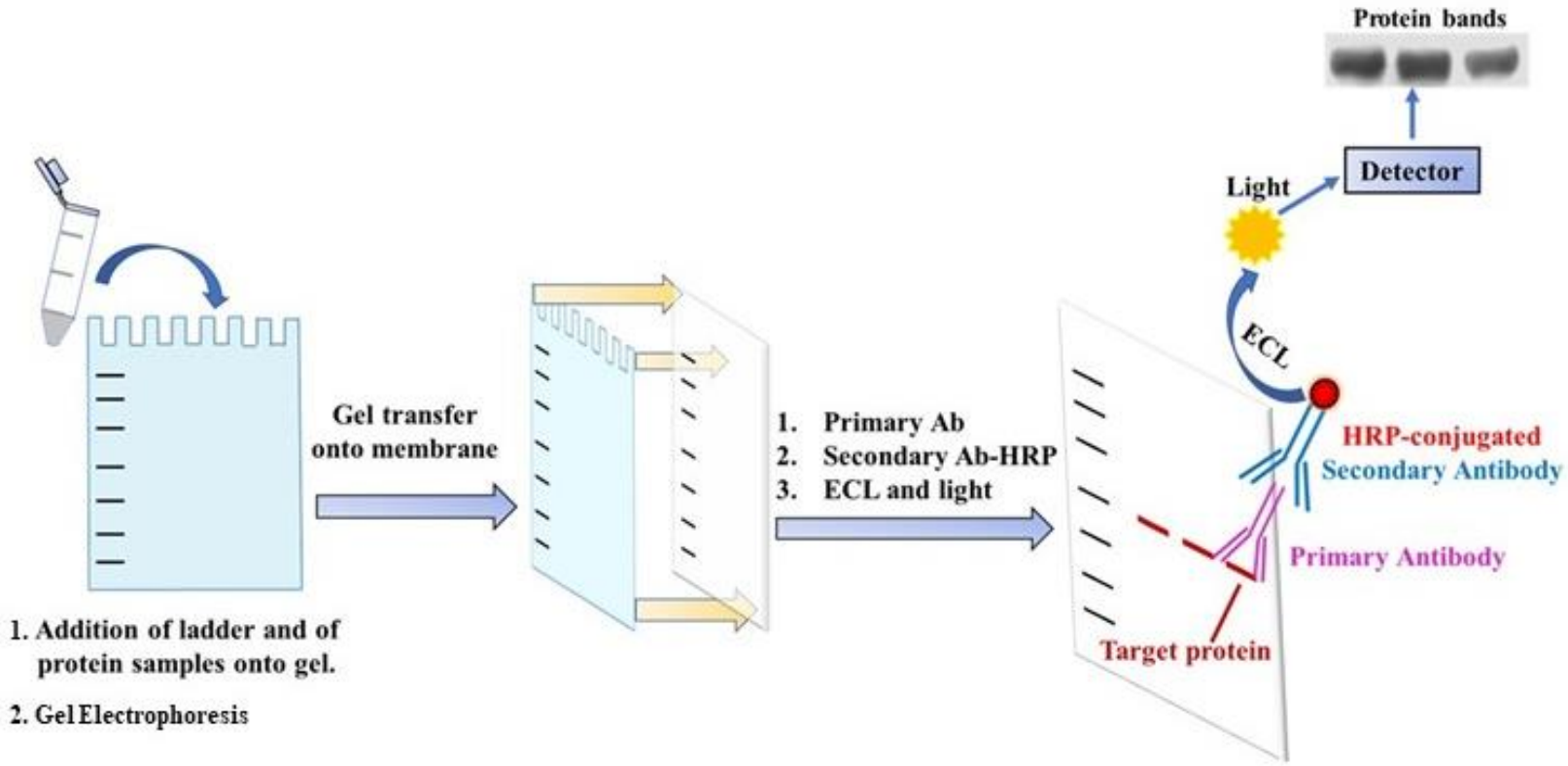


Figure 2.9 Western Blotting.

The protein lysates are loaded onto a gel and following gel electrophoresis the gel is transferred onto a membrane (nitrocellulose or PVDF) and after successful transfer a primary Ab specific to the target protein is added followed by addition of a secondary HRP-conjugated Ab which binds the primary Ab and an ECL reaction which leads to production of light and target protein detection through visualisation of the protein bands. Thorough washes are performed between each step of Ab and ECL addition.

2.2.6.2. Protein extraction and WB protocol

For the WB assays, the ciGEnCs were seeded into 6-well plates and were treated for 15 and 30 minutes (37ºC, 5% CO_2) with 10 ng/ml of TNF-α, IL-1β, IL-13 and IFN-γ and with 1 μg/ml of LPS. Following completion of treatments, the ciGEnCs were washed twice in 1 ml of cold PBS and were subsequently lysed for 10 minutes on ice, in 180 μl of RIPA buffer including 1% protease inhibitor cocktail. The samples were scraped, collected into 1.5 ml tubes and were sonicated for 10 minutes to ensure complete lysis. Subsequently, the protein samples were centrifuged at 11,000 xg for 10 minutes, the supernatants were collected and transferred into new 1.5 ml tubes.

The protein concentration of each sample was determined using the Pierce™ BCA Protein Assay Kit. The BCA assay is a biochemical method for colorimetric protein detection and quantitation[337]. It is based on the creation of an alkaline environment promoting the chelation of cupric ions (Cu^{2+}) with peptides of the protein samples leading to Cu^{2+} reduction to cuprous ions (Cu^{1+}), followed by BCA reaction with Cu^{1+} inducing the generation of an intense purple colour[337]. The assay was carried out in 96-well plates. The BSA protein standards starting from 2 mg/ml of stock BSA (8 standards, range**:** 2000 μg/ml – 125 μg/ml) were prepared as shown on the table below **(Table 2.8)**, using RIPA buffer as diluent and were used for the generation of the standard curve**.**

Standards	**Composition**
A (2000 µg/ml)	300 µl stock BSA
B (1500 µg/ml)	375 µl stock BSA + 125 µl RIPA buffer
C (1000 µg/ml)	325 µl stock BSA + 325 µl RIPA buffer
D (750 µg/ml)	325 µl B + 175 µl RIPA buffer
E (500 µg/ml)	325 µl C + 325 µl RIPA buffer
F (250 µg/ml)	325 µl E + 325 µl RIPA buffer
G (125 µg/ml)	100 µl G + 400 µl RIPA buffer
Blank	RIPA buffer

Table 2.8 BCA assay BSA standards.

All standards and protein samples were added in duplicates (10µl per well) on the 96-well plates and subsequently 200 µl of the BCA reagent (consisting of 50 parts of reagent A and 1 part of reagent B) were added on each well. The plates were then placed on a plate shaker for 30 seconds and were incubated at 37°C for 30 minutes. Following completion of the 30-minute incubation, the plates were immediately read on a plate reader at 562 nm. The standard curve (**Figure 2.10**) was generated by plotting the average absorbance values (y axis) against the standard protein concentrations (x axis) and the unknown concentrations of the protein samples were interpolated from the standard curve.

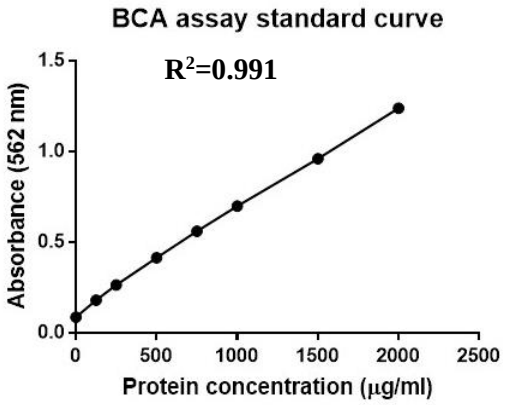


Figure 2.10 Example of BCA standard curve.

For the WB experiments, 15 μg of protein were diluted in NuPAGE LDS Sample buffer at a final 1x concentration with 50μM DTT, as a reducing agent. The proteins were boiled for 5 minutes at 90^{o}C and were loaded in 12% 50μl 10-well precast SDS-PAGE gels and protein gel electrophoresis took place into vertical electrophoresis cells filled with 1x running buffer prepared from water-diluted 10x running buffer. Gels were run at 95 V and at the end of the electrophoresis, were transferred into PVDF membranes using the Trans-Blot Turbo Transfer System. At the end of the transfer, each PVDF membrane was blocked for at least 1 hour in 5% non-fat dry milk diluted in 1x TBS-T. 1x TBS-T was prepared via diluting in water the 10x TBS stock solution and adding 0.1% Tween-20.

All antibodies were diluted in 1x TBS-T and 5% non-fat dry milk. At the end of 1-hour blocking, the primary antibodies were added onto the membranes overnight, at 4^{o}C and after completion of the primary Ab incubation each membrane was washed with TBS-T three times, with a duration of 5 minutes each. The membranes were then incubated with the secondary antibodies for 1 hour at room temperature. After the final

TBS-T washes, protein detection was achieved using ECL reagents (5-minute incubation). Li-COR software was used for protein band densitometry.

Each membrane was used for multiple protein detection, therefore, after the end of each ECL detection procedure the membranes were washed twice for 20 minutes each time with mild stripping buffer followed by two 10-minute washes with PBS and three 5-minute washes TBS-T. By the end of the stripping process, the membranes were ready to be re-blocked for detection of a different protein. Beta-actin (β-actin) was used as a loading control for reference on each gel.

2.2.7. Immunofluorescence (IF)

2.2.7.1. Basic principles of IF and indirect IF

IF is a cell imaging technique utilising fluorochrome-conjugated antibodies to detect an antigen of interest in a fixed sample and to assess its cellular abundance and distribution; visualisation and detection of the antigen of interest subsequently occurs via utilisation of a fluorescent microscope (such as a confocal or epifluorescence microscope)[338,339].

IF can be either direct or indirect depending on whether the primary Ab against the target-antigen is directly conjugated to a fluorophore (direct IF), or whether a secondary fluorophore-conjugated Ab is bound to the primary Ab which, this time, is unconjugated (indirect IF)[338,339]. (**Figure 2.11)**. Compared to the direct IF method, indirect IF can further enhance the fluorescent signal although it adds further complexity to the assay. When an intracellular protein or antigen of interest needs to be detected via IF, the cells need to be rendered permeable prior to Ab treatments.

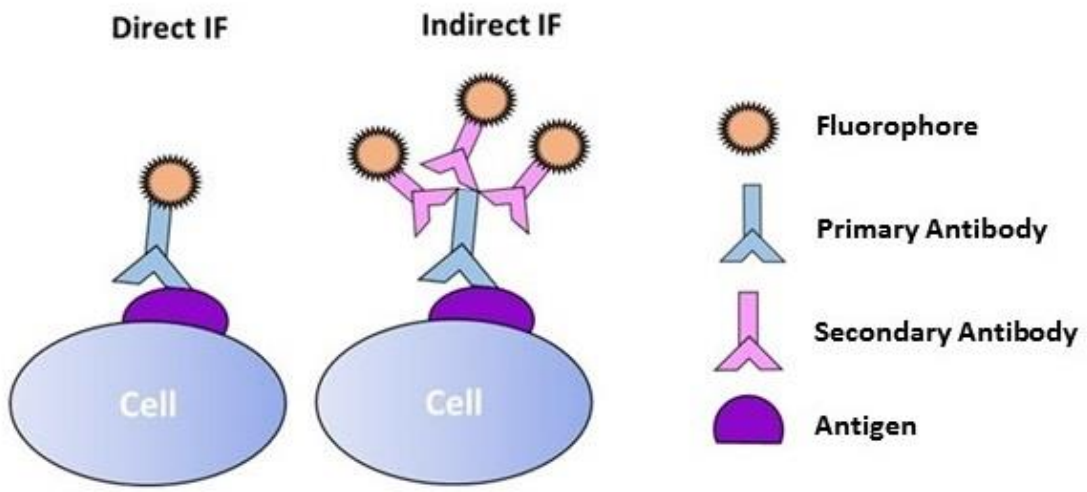


Figure 2.11 Immunofluorescence (IF).

Schematics of direct (fluorophore-conjugated primary Ab) versus indirect IF (fluorophore-conjugated secondaryAb against the primary Ab).

2.2.7.2. IF protocol

Transcription factor activation and subsequent nuclear translocation was assessed using IF. Following 30-minute incubations with 10 ng/ml of TNF-α, IL-1β and IFN-γ and with 1 μg/ml of LPS in 12-well plates, ciGEnC plates were placed on ice and cells washed three times (3x) with ice-cold PBS and were fixed in 4% formaldehyde for 30 minutes. Subsequently, ciGEnCs were washed 3x with PBS (at room temperature) and were permeabilised using 0.4% Triton-X-100 for 10 minutes. The cells were washed with PBS (3x, room temperature) and then, non-specific binding was blocked using PBS-1% BSA for 1 hour at room temperature. By the end of 1-hour incubation in PBS-BSA the cells were washed with PBS 3x (room temperature). Primary antibodies against human NF-κB, Signal transducer and activator of transcription (STAT)-1 and STAT-2 were added into plate wells and incubated at 4^{o}C overnight on a plate rocker. Following overnight incubation, ciGEnCs were washed 3x with PBS (room temperature) and were incubated with fluorescence-conjugated secondary antibodies and 1 μg/ml of DAPI, for

2 hours at room temperature. Cells were visualised immediately, using the EVOS FLoid Cell Imaging Station (ThermoFisher Scientific).

2.2.7.3. Image Analysis using ImageJ

Image analysis to determine nuclear translocation of the signal in response to cytokine or LPS treatment was performed using Fiji (ImageJ) and particularly the Coloc2 algorithm[340]. For every group of treatments, the Pearson's r-values for every single cell, representing the degree of nuclear colocalisation between DAPI and A488 or A568, were obtained.

2.2.8. Statistical analyses

Statistical analyses of all data were performed using GraphPad Prism 4.0 software. Data are expressed as median values. Multiple comparisons were made using Kruskal-Wallis or Friedman non-parametric test with Dunn's post-hoc test. Potential correlations between increasing cytokine concentrations and pro-inflammatory protein gene expression was explored using the non-parametric Spearman correlation test. For the existence of a positive correlation, the value for the correlation coefficient (r) had to be between 0.7 and 1.

For the IF experiments, for nuclear co-localisation to be statistically significant, the r-values should range between 0.7 to 1. Therefore, in all the statistical analysis diagrams a horizontal dashed line starting from the 0.7 value on the y-axis separates the cells of each treatment in two groups; the low r-value group for cells displaying moderate to low degree of nuclear co-localisation and the high r-value group for cells displaying moderate to high degree of nuclear co-localisation.

2.2.9. Summary

In this chapter, a detailed presentation of the principles and protocols of all the methods and techniques used to address the specific aims and objectives of this project outlined in section 1.8 has been provided.

3. Chapter 3: Identifying the key pro-inflammatory stimuli in glomerular endothelial cell-mediated inflammation

Work from this chapter has been published in Scientific Reports[341] (see Appendix D).

3.1. Introduction

LN is a complex disease, with one of its main features being the presence of various pro-inflammatory cytokines in the kidney microenvironment that significantly contribute to chronic inflammation induced GFB damage (see section 1.4). This can cause impaired blood plasma filtration resulting in proteinuria and haematuria, clinically detected via examination of patients' urine samples[342,343]. Although there is limited knowledge about the involvement of the human GEnCs in LN inflammation and production of pro-inflammatory mediators, strong evidence exists that HUVECs[344,345] and human brain microvascular endothelial cells (HBMECs)[346,347], when cultured *in vitro* under appropriate pro-inflammatory stimuli such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and lipopolysaccharide (LPS), elicit a robust inflammatory response. This response involves nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and production of adhesion molecules and cytokines. Thus, uncovering the role of human GEnCs, could provide further insight into LN pathogenesis.

3.2. Aim

To assess the pro-inflammatory profile of the human GEnCs using an *in vitro* model of human ciGEnCs.

3.2.1. Objectives

Objective 1: To determine the effects of pro-inflammatory cytokines involved in LN in ciGEnC expression of novel urinary biomarkers for LN and pro-inflammatory protein production, and to determine which of these cytokines have the most prominent effect.

To address this objective, an *in vitro* model of LN was developed using human ciGEnCs (see section 2.2.2) that were exposed to a pro-inflammatory environment, including cytokines involved in LN (TNF-α, IL-1β, IL-6, IL-13, IFN-α, IFN-γ, VEGF) and LPS. Subsequently, the ciGEnC pro-inflammatory phenotype was investigated via testing the production, surface expression and secretion of pro-inflammatory proteins; adhesion molecules, co-stimulatory molecules, urinary biomarkers, blood cell growth factors, cytokines and chemokines, all involved in binding and recruitment of immune cells to inflamed tissues.

O**bjective 2:** To test whether the pro-inflammatory stimulated ciGEnCs activate important pro-inflammatory transcription factors.

To address this objective, the ciGEnCs were treated with TNF-α, IL-1β and IFN-γ (being the mediators from objective 1 that elicited the most robust responses) and the induction of transcription factor nuclear translocation was tested.

Objective 3: To assess the viability of ciGEnCs after pro-inflammatory stimulation.

To address this objective, the ciGEnCs were treated with the cytokines found to have the most prominent effect on ciGEnC pro-inflammatory activation as well as LPS, and their apoptosis/necrosis levels were tested.

3.2.2. Chapter hypothesis

Under *in vitro* stimulation with pro-inflammatory cytokines and with LPS the ciGEnCs will exhibit a robust pro-inflammatory phenotype including activation of pro-inflammatory transcription factors, and increased secretion of cytokines, chemokines and blood cell growth factors and surface expression of adhesion and co-stimulatory molecules. This phenotype will be an active response to pro-inflammatory stimuli and not an event occurring due to ciGEnC death.

3.3. Materials and Methods

3.3.1. ciGEnC preparation and treatment

The fully differentiated ciGEnCs were seeded on 6-well plates for apoptosis assays (Annexin V-FITC and PI staining), 12-well plates for adhesion molecule (VCAM-1, ICAM-1), and co-stimulatory molecule (PD-L1, ICOS-L) FC assays, 6-well plates for nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (Iκ-Bα) WB experiments, 12-well plates for SYBR Green real-time PCR (*MCP-1*, *VCAM-1*, *TF* and *L-PGDS* mRNA expression), ELISA assays (MCP-1, sVCAM-1, IL-6, IL-8, IL-10, M-CSF, GM-CSF, IP-10, MIP-1α, TNF-α, IFN-γ) and IF assays (NF-κB, STAT-1, STAT-2) and on 24-well plates for mRNA concentration response real-time PCR assays.

Subsequently, ciGEnCs were incubated at 37^{o}C (with 5% CO_2) for 15 minutes, 30 minutes, 4 hours and 24 hours with 10 ng/ml of human recombinant IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-13 and VEGF individually and in combination. The potential effect of bacterial endotoxin inflammation was also examined via 1 μg/ml of LPS treatment. Untreated ciGEnCs cultured in media without addition of pro-inflammatory cytokines or LPS, were included at all experiments for the identification of potential treatment-

induced changes in mRNA, protein and transcription factor levels compared to basal ciGEnC levels.

3.3.2. qRT-PCR for the expression of urinary biomarkers

The ciGEnCs were stimulated for 24 hours with IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-13 and VEGF alone and in combination and with LPS. Subsequently, the cells were washed and immediately lysed with Trizol to perform Trizol-chloroform RNA extraction. RNA was transcribed to cDNA and qRT-PCR was performed to investigate the expression levels of the urinary biomarkers for active LN biomarkers; AGP, CP, L-PGDS, TF, NGAL, MCP-1, VCAM-1.

3.3.3. ELISA assays

The secretion of MCP-1, sVCAM-1, IL-6, IL-8, IL-10, M-CSF, GM-CSF, MIP-1α, IP-10, TNF-α and IFN-γ was tested with single ELISAs or a Luminex multiplex ELISA assay, utilising conditioned media from ciGEnCs treated for 24 hours with cytokines (as above) and LPS. Conditioned media from IL-6 and VEGF treatments were excluded as they induced no change in the mRNA expression of the urinary biomarkers. However, IL-6 and VEGF were still included in the combined cytokine treatment (All).

3.3.4. Concentration response for TNF-α, IL-1β, IL-13 and IFN-γ

A concentration response experiment was performed for TNF-α, IL-1β, IL-13 and IFN-γ to ensure that the 10 ng/ml used for ciGEnC stimulation was the lowest *in vitro* concentration able to induce a robust increase in the expression of pro-inflammatory genes. The ciGEnCs were treated for 24 hours with TNF-α, IL-1β, IL-13 and IFN-γ with 10 ng/ml being the highest concentration used to perform ten-fold serial dilutions, with 10 pg/ml being the lowest concentration used (10 ng/ml → 1 ng/ml → 100 pg/ml → 1

pg/ml). Following completion of the 24-hour treatments, the conditioned media were removed, cells were washed with PBS and were immediately lysed with Trizol to carry out a Trizol-chloroform RNA extraction and, subsequently, cDNA transcription that was used for qRT-PCR assays.

The TNF-α-, IL-1β-, IL-13- and IFN-γ-stimulated ciGEnCs were found to incrementally upregulate the mRNA expression of pro-inflammatory genes following stimulation with increasing cytokine concentrations, with the 10 ng/ml treatments inducing the most robust effect.

A positive correlation was observed between increasing concentrations of TNF-α and *MIP-1α* mRNA (r=0.9, p=0.08) and a statistically significant correlation for increasing concentrations of TNF-α and *M-CSF* (r=1, p=0.02) mRNA levels (**Figure 3.1**). A positive correlation, although not statistically significant, was also observed between increasing doses of TNF-α and *IL-8* (r=0.7, p=0.23) and *GM-CSF* (r=0.7, p=0.23) mRNA levels (**Figure 3.1**). No strong correlation was found between increasing TNF-α concentrations and *MCP-1* (r=0.6, p=0.35) and *VCAM-1* (r=0.5, p=0.45) mRNA levels, however the highest TNF-α concentration (10 ng/ml) induced the expression of considerably higher mRNA levels compared to untreated ciGEnCs (**Figure 3.1**).

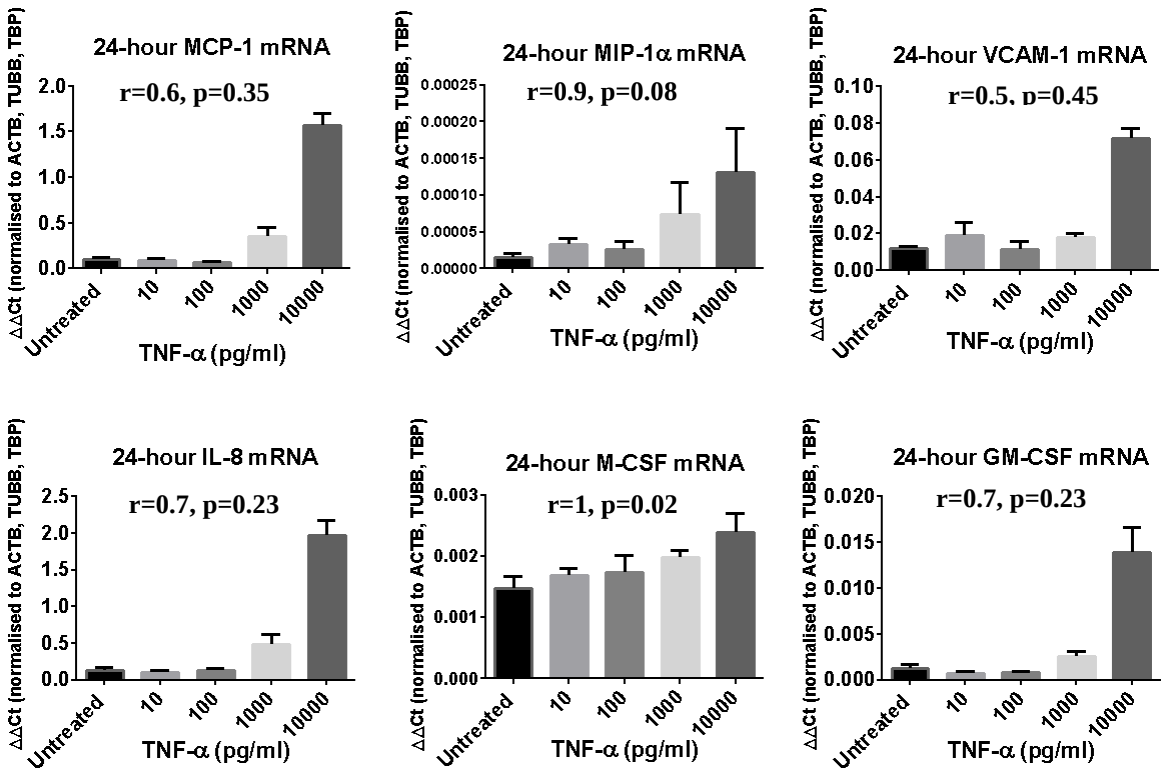


Figure 3.1 24-hour TNF-α concentration response assays.

Changes in the mRNA expression levels of MCP-1, MIP-1α, VCAM-1, IL-8, M-CSF and GM-CSF following treatments with increasing concentrations of TNF-α. N=3-4/group. Data are presented as (Mean +/- SEM) and correlations are analysed using non-parametric Spearman's test. HK: ACTB, TUBB, TBP.

From the IL-1β concentration response experiments, a positive correlation occurred between increasing concentrations of IL-1β and mRNA levels of *MCP-1* (r=0.9, p=0.08), and a statistically significant correlation was observed for increasing IL-1β concentrations and *MIP-1α* (r=1, p=0.02), *IL-6* (r=1, p=0.02), *IL-8* (r=1, p=0.02), *M-CSF* (r=1, p=0.02) and *GM-CSF* (r=1, p=0.02) mRNA levels (**Figure 3.2**).

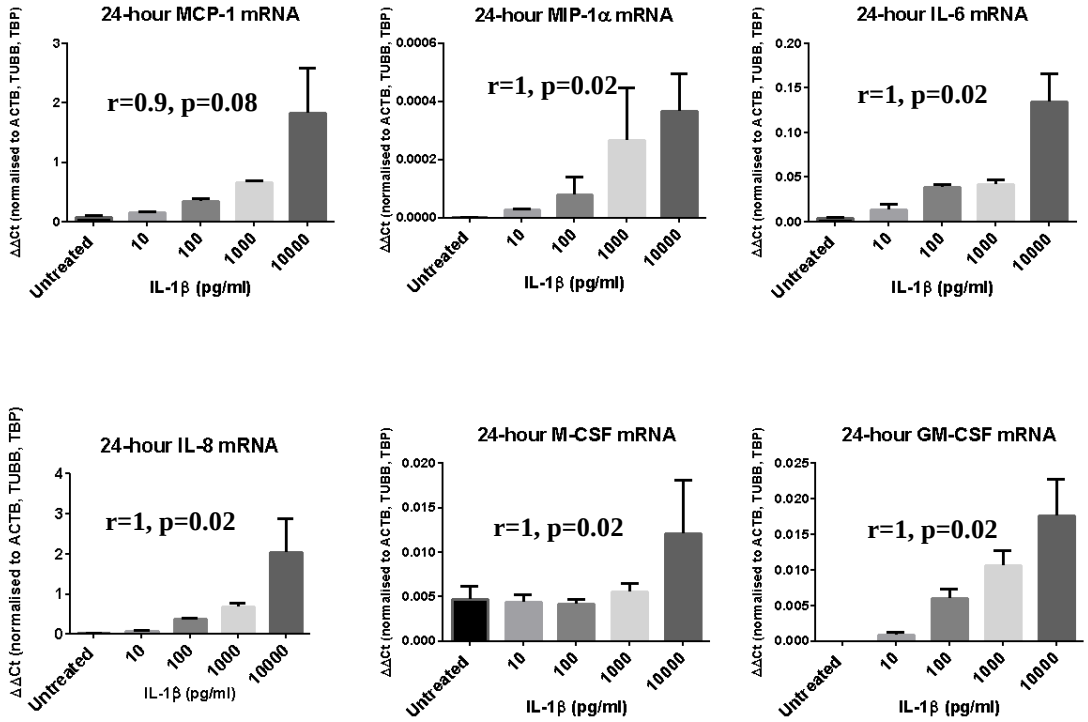


Figure 3.2 24-hour IL-1β concentration response assays.

Changes in the mRNA expression levels of MCP-1, MIP-1α, IL-6, IL-8, M-CSF and GM-CSF following treatments with increasing concentrations of IL-1β. N=3-4/group. Data are presented as (Mean +/- SEM) and correlations are analysed using non-parametric Spearman's test. HK: ACTB, TUBB, TBP.

A significant positive correlation (r=1, p=0.02) was also observed between increasing concentrations of IL-13 and *VCAM-1* mRNA levels (**Figure 3.3**).

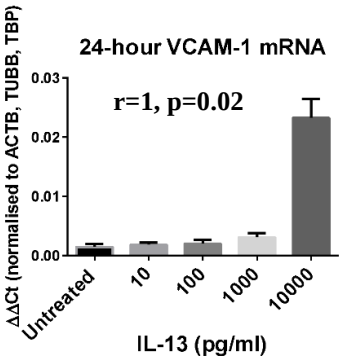


Figure 3.3 24-hour IL-13 concentration response assays..

Changes in the mRNA expression levels of VCAM-1 following treatments with increasing concentrations of IL-13. N=3-4/group. Data are presented as (Mean +/- SEM) and correlations are analysed using non-parametric Spearman's test. HK: ACTB, TUBB, TBP

Finally, a positive correlation (r=0.9, p=0.08) was observed for increasing concentrations of IFN-γ and *IP-10* mRNA levels but no correlation was observed between IFN-γ and *IL-10* mRNA levels which were generally lowly expressed (**Figure 3.4**).

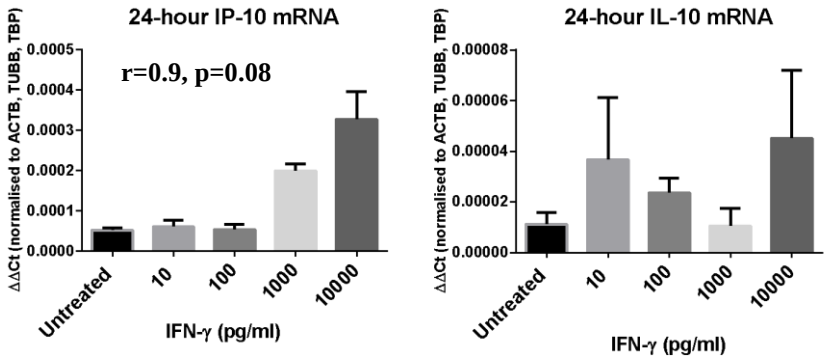


Figure 3.4 24-hour IFN-γ concentration response assays. .

Changes in the mRNA expression levels of IP-10 and IL-10 following treatments with increasing concentrations of IFN-γ. N=3-4/group. Data are presented as (Mean +/- SEM) and correlations are analysed using non-parametric Spearman's test. HK: ACTB, TUBB, TBP

3.3.5. FC for leukocyte adhesion molecules, T cell co-stimulatory molecules and cytokine receptors

The ciGEnCs were treated for 4 or 24 hours with TNF-α, IL-1β, IL-13, IFN-γ and LPS. Following completion of treatments, the conditioned media were removed, cells were washed with PBS, trypsinised and pelleted at 300 xg for 5 minutes and were then resuspended in PBS with 0.1% BSA and stained for 30 minutes on ice with antibodies specific for human E-/P-selectin, VCAM-1, ICAM-1, PD-L1 and ICOS-L, prior to flow cytometric analysis.

3.3.6. ciGEnC viability

The ciGEnCs were treated for 24 hours with TNF-α, IL-1β, IL-13, IFN-γ and LPS. Subsequently, the ciGEnCs were trypsinised, centrifuged at 300 xg for 5 minutes and

the cell pellets were resuspended in HBSS and initially stained with Annexin V – FITC (15 minutes on ice) and then with PI (5 minutes, room temperature, dark) to perform a flow cytometric apoptosis assay.

3.3.7. Western Blotting for Iκ-Bα

ciGEnCs were stimulated for 15 and 30 minutes with TNF-α, IL-1β, IL-13, IFN-γ and LPS, washed with ice-cold PBS and immediately after the washes were lysed using ice-cold RIPA buffer with protease inhibitor cocktail (15 minutes on ice, with occasional swirling). Cell lysates were collected, sonicated and centrifuged for 10 minutes at approximately 11,000 xg, Supernatants were collected, and protein concentration of each sample was determined with BCA assay. From each sample equal protein concentrations were loaded onto gels for electrophoresis, using sample buffer together with DTT as previously described in the Materials & Methods (see section 2.2.6). Human Iκ-Bα levels were detected on the membranes, with human β-actin used as a loading control.

3.3.8. Immunofluorescence for NF-κB, STAT-1 and STAT-2

The ciGEnCs were treated for 15 and 30 minutes with TNF-α, IL-1β and LPS to investigate NF-κB nuclear translocation and with IFN-γ to investigate STAT-1 and STAT-2 nuclear translocation. Following the end of treatments cells were washed with ice-cold PBS, were fixed with formaldehyde for 20 minutes, washed with PBS and were permeabilised with Triton-X for 10 minutes. Subsequently, the cells were washed with PBS, were incubated at room temperature for at least 1 hour with PBS and 0.1% BSA and were then blocked with anti-human NF-κB, STAT-1 and STAT-2 antibodies at 4^{o}C overnight. Following overnight incubation, cells were washed with PBS, blocked for 2 hours at room temperature with secondary fluorescence-conjugated antibodies and immediately afterwards PBS was added and ciGEnCs were visualised using the EVOS

FLoid Cell Imaging Station. Image analysis took place using Fiji (Image J) as previously described within the general "Materials and Methods" (see section 2.2.7.3).

3.3.9. Statistical analyses

Changes between two different groups were studied using non-parametric Mann-Whitney test whereas changes among three or more groups were studied using Kruskal-Wallis or Friedman's test with Dunn's post-hoc test. Potential correlations (r >= 0.7) were tested using non-parametric Spearman's test.

3.4. Results

3.4.1. Inflammatory stimulation of ciGEnCs induces the production of urinary biomarkers and pro-inflammatory proteins

3.4.1.1.Investigating the mRNA expression and secretion of urinary biomarkers

ciGEnC expression of urinary biomarker mRNA was tested after 24 hours of cytokine and LPS incubation. No basal, cytokine- or LPS-induced mRNA expression levels of *AGP*, *CP*, or *NGAL* were detected. This indicated that the human GEnCs are not able to produce these biomarkers and therefore, other renal cell types or immune cells could be responsible for their production.

MCP-1 and *VCAM-1* mRNA expression levels were significantly increased following certain cytokine treatments or LPS treatment (**Figure 3.5 A-B)**. More specifically, for *MCP-1* and *VCAM-1* mRNA expression, TNF-α (3.67 [3.1-3.2], p=0.004), IL-1β (2.3 [1.2-3.1], p=0.05) and the combined cytokine treatment (All: 3.6 [3.6-5.4], p=0.001) significantly increased *MCP-1* levels compared to untreated ciGEnCs (0.17 [0.07-0.7]) (**Figure 3.5-A)** whereas IL-13 (0.62 [0.29-0.74], p=0.002) significantly upregulated *VCAM-1* expression compared to the untreated cells (0.03

[0.02-0.11]), as did the combined cytokine treatment (0.17 [0.12-0.25]), potentially due to the combined action of IL-13 with TNF-α (**Figure 3.5-B)**. The ciGEnCs were also able to express *L-PGDS* and *TF* mRNA. However, in low basal levels that were not altered by cytokine or LPS treatments (**Figure 3.5 C-D**).

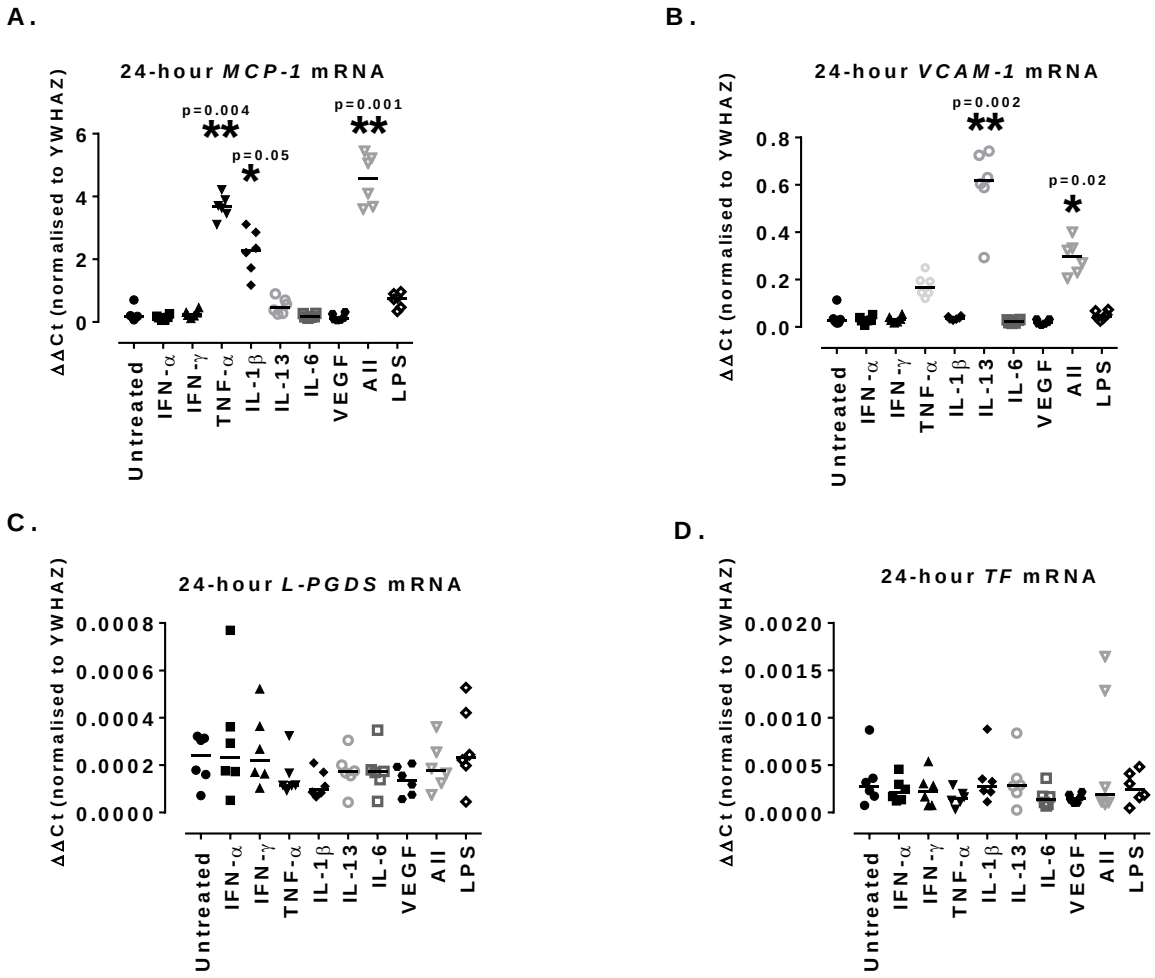


Figure 3.5 qRT-PCR for MCP-1, VCAM-1, L-PGDS and TF mRNA after 24-hour treatments with cytokines and LPS.

*A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in L-PGDS mRNA levels. D. Changes in TF mRNA levels N=5-6/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p ≤0.05, **p ≤0.01 vs untreated. HK gene: YWHAZ.*

At the protein level, the combination of all cytokines (All: 3,755pg/mL [3,159-3,989], p=0.008) led to significantly higher levels of secreted MCP-1 compared to the untreated cells (800 pg/mL [346-1,856]) (**Figure 3.6-A**), an effect also observed for LPS (2,876pg/mL [2,457-3,072], p=0.04). A significant increase in secreted levels of sVCAM-1 (**Figure 3.6-B**) was only induced by IL-13 (1,539pg/mL [731-1,718], p=0.02) compared to the untreated cells (192pg/mL [73-661]). Culture media after IL-6 and VEGF treatment were excluded from Luminex ELISA analysis due to space limitations and due to lack of mRNA induction for *MCP-1* and *VCAM-1*.

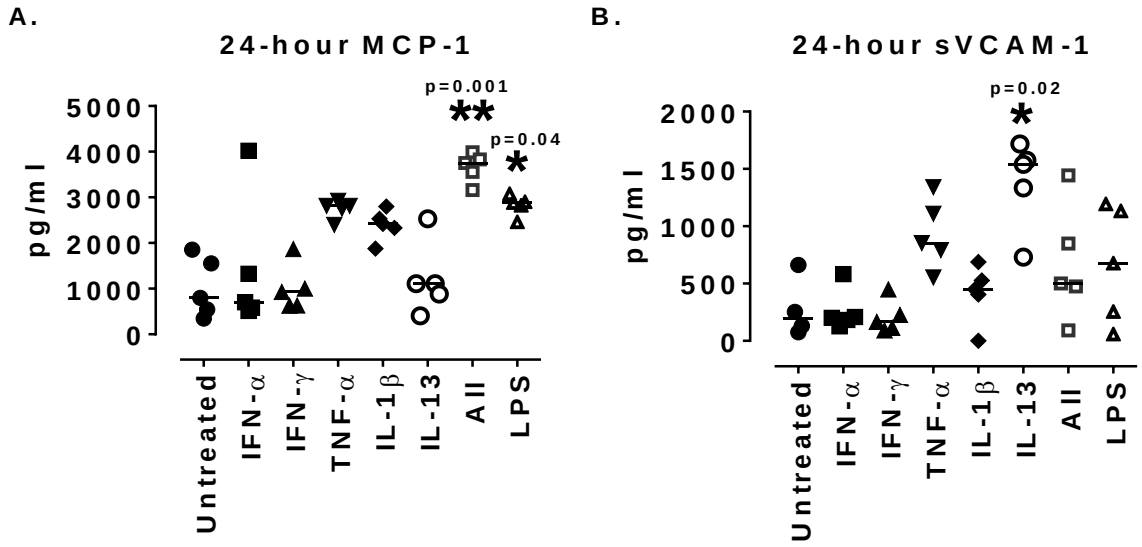


Figure 3.6 Urinary biomarker secretion after 24-hour ciGEnC-stimulation with individual cytokines, combination of all cytokines (All) and LPS.

*A. MCP-1. B. sVCAM-1. N=5-6/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05, **p≤0.01 vs untreated.*

3.4.1.2.Investigating the secretion of pro-inflammatory proteins

As the ciGEnCs substantially produce and secrete only two out of the seven identified novel urinary biomarkers for LN [15] (MCP-1 that is a chemokine and VCAM-1 that is an adhesion molecule), it was hypothesised that the role of GEnCs in the initiation and sustainability of LN renal inflammation can be related to the production and local secretion of various pro-inflammatory proteins. To address this hypothesis, changes in secretion levels of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6 and IL-10 and chemokines such as MIP-1α, IP-10 and IL-8 as well as blood cell growth factors such as GM-CSF and M-CSF, were examined via Luminex ELISA assay.

Culture media after IL-6 and VEGF treatment were excluded from Luminex ELISA analysis due to space limitations and due to lack of mRNA induction for *MCP-1* and *VCAM-1*.

Cytokine secretion following stimulation with cytokines and LPS

IFN-γ (8pg/mL [7-9], p=0.029), IL-1β (11pg/ml [7-13], p=0.011) and LPS (14pg/ml [8-21], p=0.0005) treatments significantly upregulated TNF-α secretion compared to untreated cells (3.5pg/ml [3-4]) (**Figure 3.7-A)**. The single TNF-α treatment and the combined cytokine treatment were excluded as they gave false positive results due to the presence of recombinant human TNF-α, however these treatment groups were still included in the statistical analysis. LPS treatment (28pg/ml [2-42], p=0.005) induced the secretion of significantly higher amounts of IFN-γ compared to untreated cells (1pg/ml [1-1]) (**Figure 3.7-B)**. The single IFN-γ treatment and the combined cytokine treatment were excluded from the graphs as they gave false positive results due to the presence of recombinant IFN-γ, but these groups' data were still included in the statistical analysis test.

IL-6 secretion was significantly upregulated after IL-1β (3,479pg/ml [1,599-5,273], p=0.0004) and LPS stimulation (3,323pg/ml [1,083-5,228], p=0.0007) (**Figure 3.7-C**) compared to untreated ciGEnCs (31pg/ml [11-163]). IFN-γ (18pg/ml [14-21], p=0.004), combined cytokine treatment (All: 24pg/ml [22-24], p<0.0001) and LPS (17pg/ml [8-21], p=0.009) significantly increased IL-10 secretion compared to untreated cells (0.5pg/ml [0.3-0.7]) (**Figure 3.12-D**).

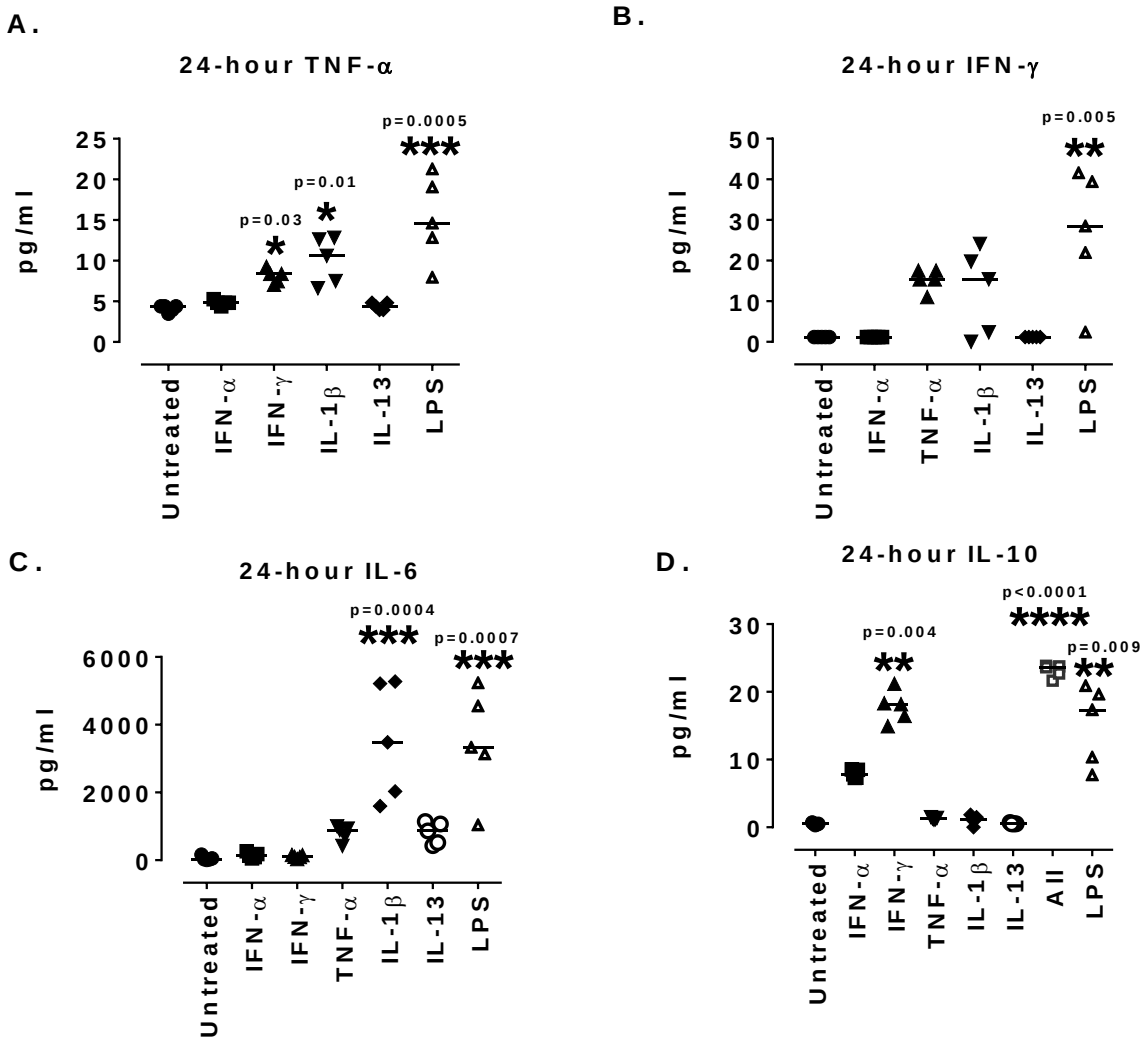


Figure 3.7. Cytokine secretion after 24-hour ciGEnC-stimulation with individual cytokines, combination of all cytokines (All) and LPS.

*A. TNF-α. B. IFN-γ. C. IL-6. D. IL-10. N=5-6/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *P≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001 vs untreated.*

Chemokine secretion following stimulation with cytokines and LPS

A trend was observed for increased IL-8 secretion after combined cytokine treatment (All; 6,127pg/mL [3,664-6,255], p=0.066) compared to untreated cells (623pg/ml [449-1,503]) (**Figure 3.8-A**). IFN-γ (3,015pg/ml [2,530-3,201], p=0.008) and combination of cytokines (All: 3,222pg/ml [3,153-3,262], p=0.0003) significantly upregulated IP-10 secretion compared to untreated cells (2pg/ml [2-4]), as did LPS (2,870pg/ml [847-3,218], p=0.013) (**Figure 3.8-B)**. TNF-α (222pg/ml [221-223], p=0.048), IL-1β (222pg/ml [213-226], p=0.049) and the combined cytokine treatment (All: 239pg/ml [233-243], p=0.0002) significantly increased the secretion of MIP-1α compared to untreated cells (209pg/ml [207-209], as did LPS (226pg/ml [215-230], p=0.011) (**Figure 3.8-C)**.

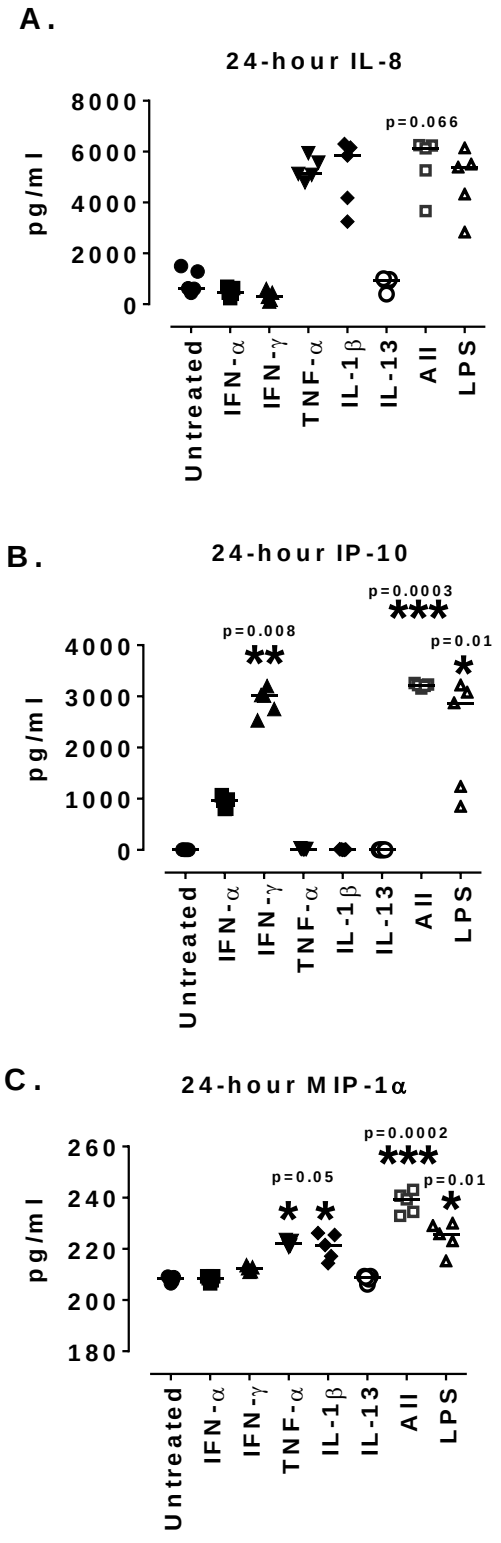


Figure 3.8 Chemokine secretion after 24-hour ciGEnC-stimulation with individual cytokines, combination of all cytokines (All) and LPS.

*A. IL-8. B. IP-10. C. MIP-1α. C. IL-6. N=5-6/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *P=0.05, **p=0.01, ***p≤0.001 vs untreated.*

Blood cell growth factor secretion following stimulation with cytokines and LPS

The combined cytokine treatment led to secretion of significantly higher amounts of M-CSF (All: 3,755pg/ml [3,159-3,989], p=0.001) compared to untreated cells (800pg/ml [346-1,856]), as did LPS (2876pg/ml [2,457-3,072], p=0.04) (**Figure 3.9-A).** IL-1β (2,284pg/ml [710-2,748], p=0.0001) and TNF-α (522pg/ml [493-722], p=0.02) significantly increased the secretion of GM-CSF compared to untreated cells (20pg/ml [17-40]), as did the combined cytokine treatment (All: 1,010pg/ml [323-1,925], p=0.002) and LPS (790pg/ml [231-1,882], p=0.008) (**Figure 3.9-B)**.

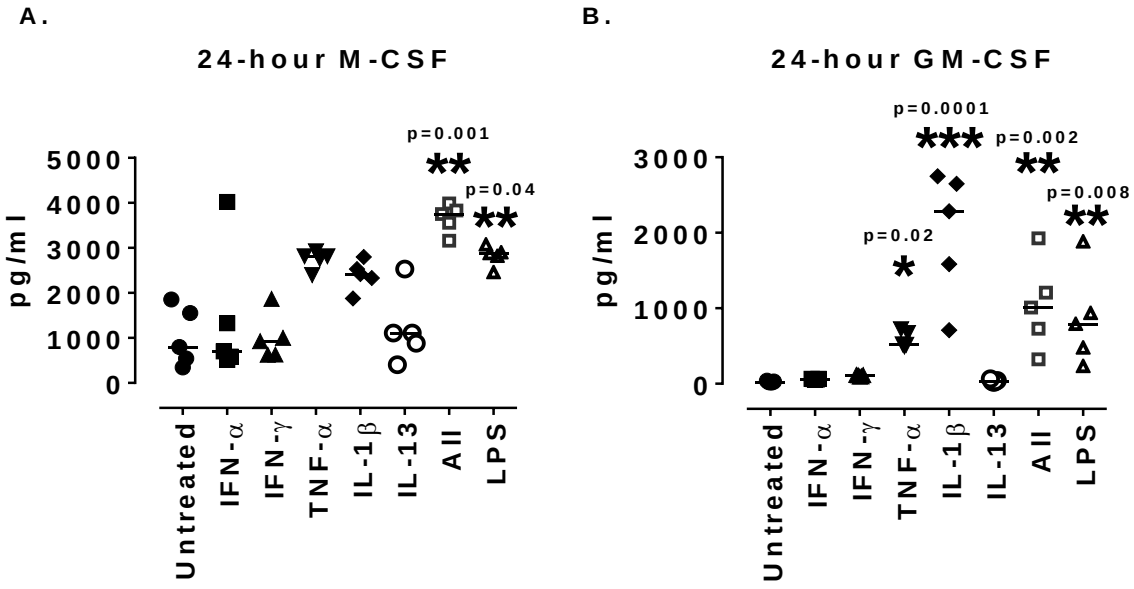


Figure 3.9 Blood cell growth factor secretion after 24-hour ciGEnC-stimulation with individual cytokines, combination of all cytokines (All) and LPS.

*A. M-CSF. B. GM-CSF. N=5-6/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05, **p≤0.01, ***p≤0.001 vs untreated.*

3.4.2. Inflammatory stimulation leads to differential expression of cellular adhesion and co-stimulatory molecules

The endothelium is responsible for the recruitment and activation of leukocytes to the glomerular space by upregulating expression of cellular adhesion and co-stimulatory molecules (see section 1.4). The role of TNF-α, IL-1β, IL-13, IFN-γ and LPS, as the five most robust *in vitro* ciGEnC stimuli among those tested for pro-inflammatory protein secretion, were assessed by determining their ability to modulate the surface expression of three leukocyte adhesion molecules and two T cell co-stimulatory molecules, known to play important roles in the inflammatory properties of the endothelia; E-/P-selectin, VCAM-1, ICAM-1, PD-L1 and ICOS-L.

The fluorescence intensity signals were measured, and the geometric mean of the fluorescence intensity was calculated for each treatment condition and the geometric mean fluorescence intensity value for isotype control was subtracted from these values. Isotype control geometric mean values higher than those of untreated/treatment groups, indicated lack of surface expression (demonstrated as 0 values).

3.4.2.1.E-/P-selectin surface expression following TNF-α and IL-13 stimulation

The surface expression of E-/P-selectin by ciGEnCs was tested following 4- and 24-hour treatments with TNF-α and IL-13 alone, or in combination. Basal expression of E-/P-selectin was low at 4 hours (**Figure 3.10-A**) and almost negligible at 24 hours (**Figure 3.10-B**). Furthermore, no statistically significant changes were observed at either 4 or 24 hours after the cytokine treatments when they were compared to the basal levels of untreated ciGEnCs.

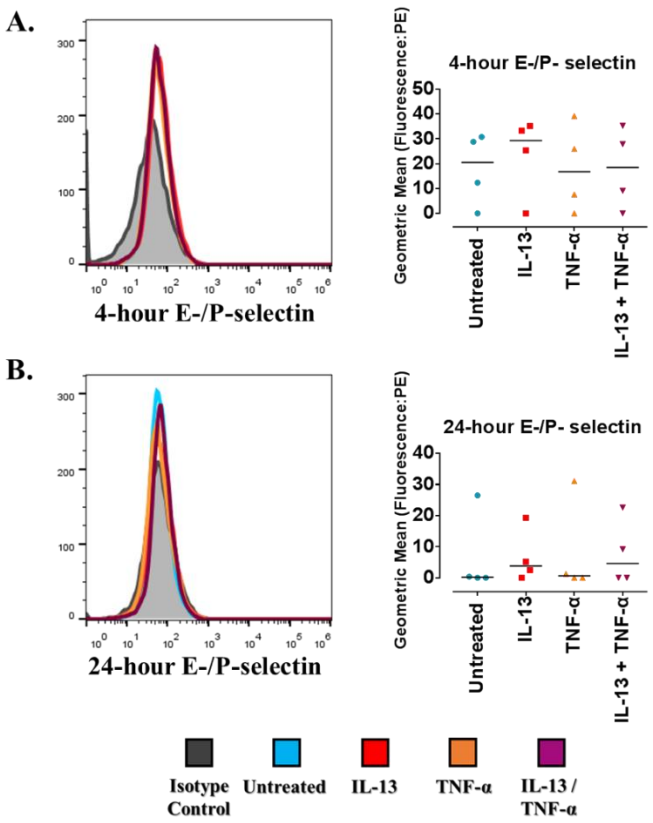


Figure 3.10 E-/P-selectin surface expression following 4- and 24-hour stimulation with TNF-α and IL-13 alone, or in combination.

A. 4-hour E-/P-selectin surface expression following IL-13 and TNF-α treatments. B. 24-hour E-/P-selectin surface expression following IL-13 and TNF-α treatments. Data presented as (median [range]) are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS.

3.4.2.2.VCAM-1 and ICAM-1 surface expression following stimulation with cytokines and LPS

TNF-α and IL-13, alone or in combination, had no significant effect on VCAM-1 (**Figure 3.11-A**) surface expression at 4 hours. At 24 hours, TNF-α (14 [7-86], p=0.008) and more markedly the combined action of IL-13 and TNF-α (IL-13+TNF-α: 54 [11-157], p=0.0007) significantly upregulated surface expression of VCAM-1 compared to untreated ciGEnCs (3 [0-4]) (**Figure 3.11-B**). At 4 hours, LPS (10 [6-38], p=0.01) significantly increased the surface expression of VCAM-1 compared to untreated

ciGEnCs (0 [0-0)] (**Figure 3.11-C**). At 24 hours, IL-1β, IFN-γ and LPS exhibited no effect on surface VCAM-1 expression (**Figure 3.11-D**).

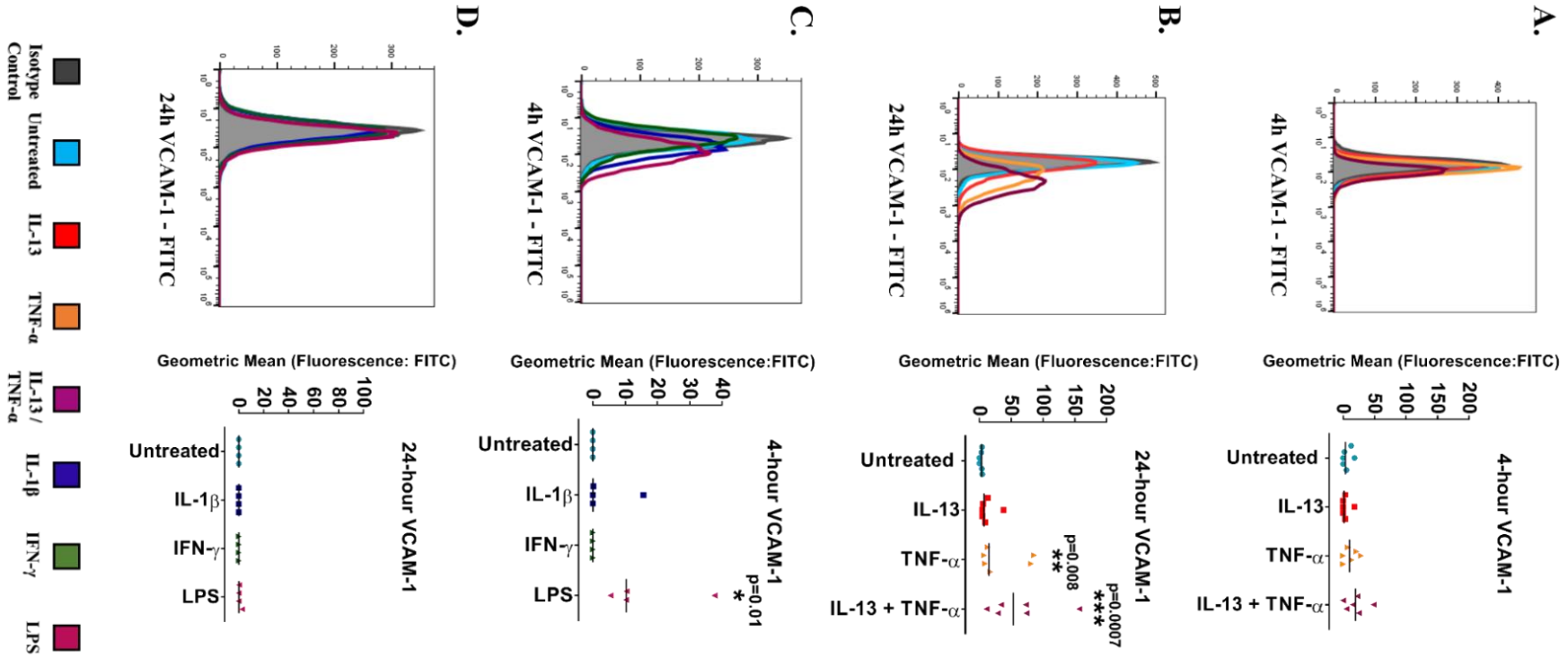


Figure 3.11 VCAM-1 surface expression following 4- and 24-hour stimulation with cytokines and LPS.

*A. 4-hour VCAM-1 surface expression following IL-13 and TNF-α treatments. B. 24-hour VCAM-1 surface expression following IL-13 and TNF-α treatments. C. 4-hour VCAM-1 surface expression following IL-1β, IFN-γ and LPS treatments. D. 24-hour VCAM-1 surface expression following IL-1β, IFN-γ and LPS treatments. N=4-6/group. Data presented as (median [range]) were analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05, **p≤0.01, ***p≤0.001 vs untreated.*

TNF-α and IL-13, alone or in combination, had no significant effect on VCAM-1 (**Figure 3.12-A**) surface expression at 4 hours. At 24 hours, TNF-α alone (19,789 [13,348-25,897], p=0.009) significantly upregulated surface expression of the constitutively expressed ICAM-1 compared to untreated ciGEnCs (6,609 [3,984-9,632]) (**Figure 3.12-B**). IL-13 (5,836 [4,684-10,747], p=NS) had no effect on ICAM-1 surface levels. IL-1β, IFN-γ and LPS treatments had an effect predominantly on ICAM-1. IL-1β (5,770 [4,874-9,777], p=0.04) and LPS (7,323 [5,593-9,734], p=0.007) significantly increased surface ICAM-1 expression at 4 hours compared to untreated ciGEnCs (2,278 [1,415-3,265]) (**Figure 3.12-C**). This effect was not only sustained for up to 24 hours of continuous incubation, but also both IL-1β (6,562 [5,953-9,673], p=0.004) and LPS (7,997 [5,497-12,334], p=0.002) further enhanced ICAM-1 surface expression compared to untreated ciGEnCs (1,727 [1,339-3,161) (**Figure 3.12-D**).

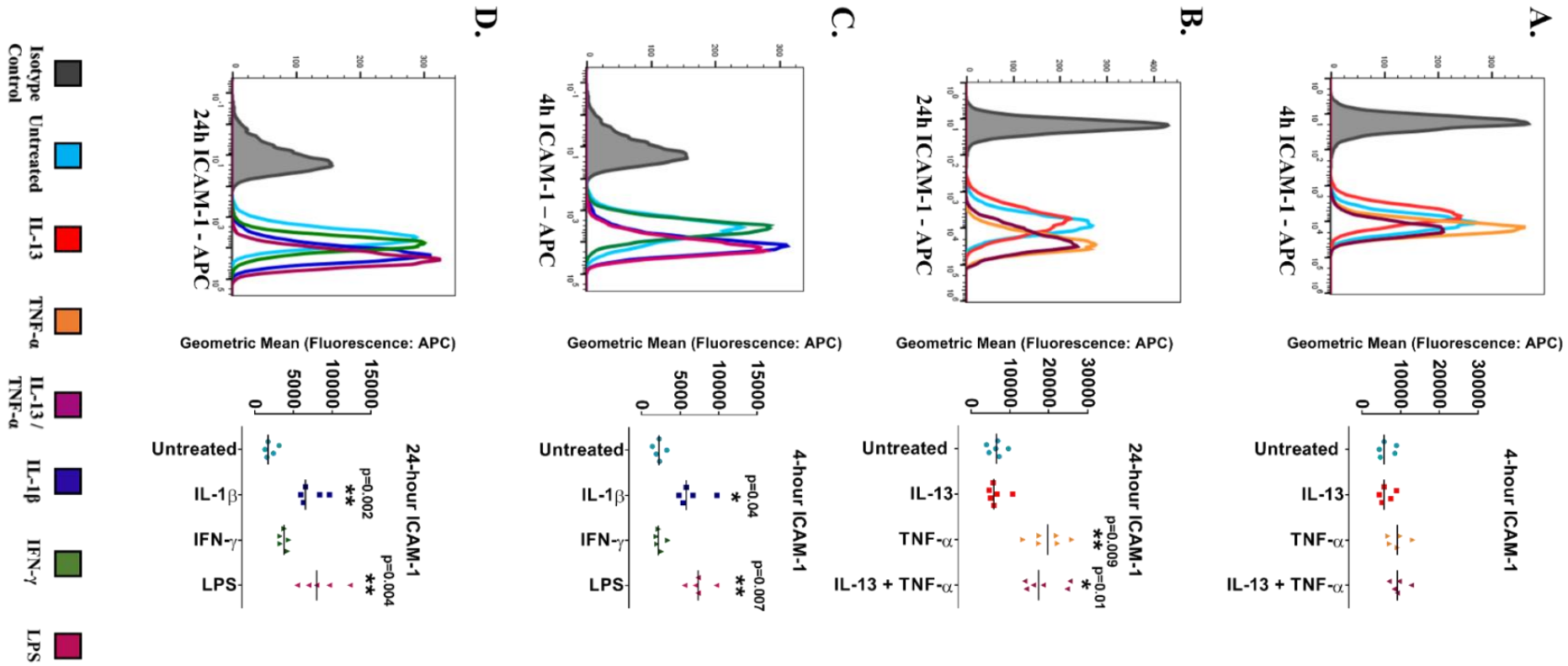


Figure 3.12 ICAM-1 surface expression following 4- and 24-hour stimulation with cytokines and LPS.

*A. 4-hour ICAM-1 surface expression following IL-13 and TNF-α treatments. B. 24-hour ICAM-1 surface expression following IL-13 and TNF-α treatments. C. 4-hour ICAM-1 surface expression following IL-1β, IFN-γ and LPS treatments. D. 24-hour ICAM-1 surface expression following IL-1β, IFN-γ and LPS treatments. N=4-6/group. Data presented as (median [range]) were analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05, **p≤0.01 vs untreated.*

3.4.2.3.PD-L1 and ICOS-L surface expression following stimulation with cytokines and LPS

IFN-γ had no effect on ICAM-1 or VCAM-1 but at 24 hours significantly increased the surface expression of PD-L1 (IFN-γ: 3549 [2199-3828], p=0.02), a negative T cell co-stimulatory molecule[348], compared to untreated ciGEnCs (1763 [793-2067]) (**Figure 3.13-B**). Finally, surface ICOS-L expression was significantly increased at 24 hours only by IL-1β (380.4 [365.4-401.4], p=0.035) compared to untreated ciGEnCs (55.1 [52.1-8.6]) (**Figure 3.13-D**).

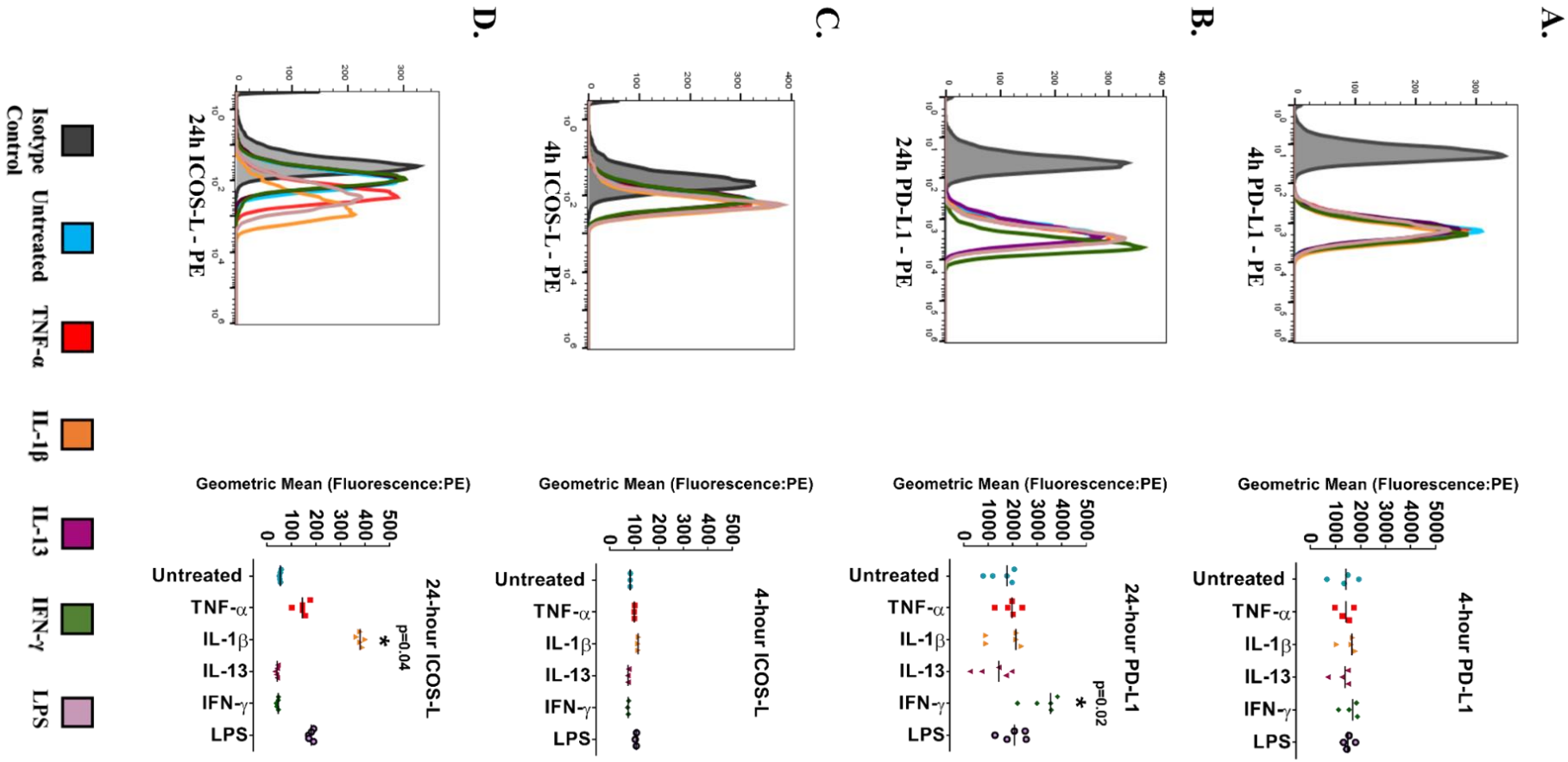


Figure 3.13 PD-L1 and ICOS-L surface expression following 4- and 24-hour stimulation with TNF-α, IL-1β, IL-13, IFN-γ and LPS.

*A. 4-hour surface PD-L1 expression. B. 24-hour surface PD-L1 expression. C. 4-hour surface ICOS-L expression. D. 24-hour surface ICOS-L expression. N=3-5/group. Data presented as (median [range]) were analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05 vs untreated.*

3.4.3. Inflammatory protein production is not associated with increased cellular death

The apoptotic/necrotic levels of ciGEnCs after 24-hour incubation with TNF-α, IL-1β, IL-13, IFN-γ and LPS were tested, in order to elucidate whether the production of pro-inflammatory proteins was an active process, or, a passive process due to cell death.

The majority of ciGEnCs remained viable (Anx V-/PI-) after 24-hour treatment with cytokines and LPS similarly to the untreated ciGEnCs (**Figure 3.14-A**). No significant changes were observed in the levels of apoptotic (Anx V+/PI-) (**Figure 3.14-A**) and late apoptotic/necrotic (Anx V+/PI+) ciGEnCs (**Figure 3.14-A**) among the different treatments.

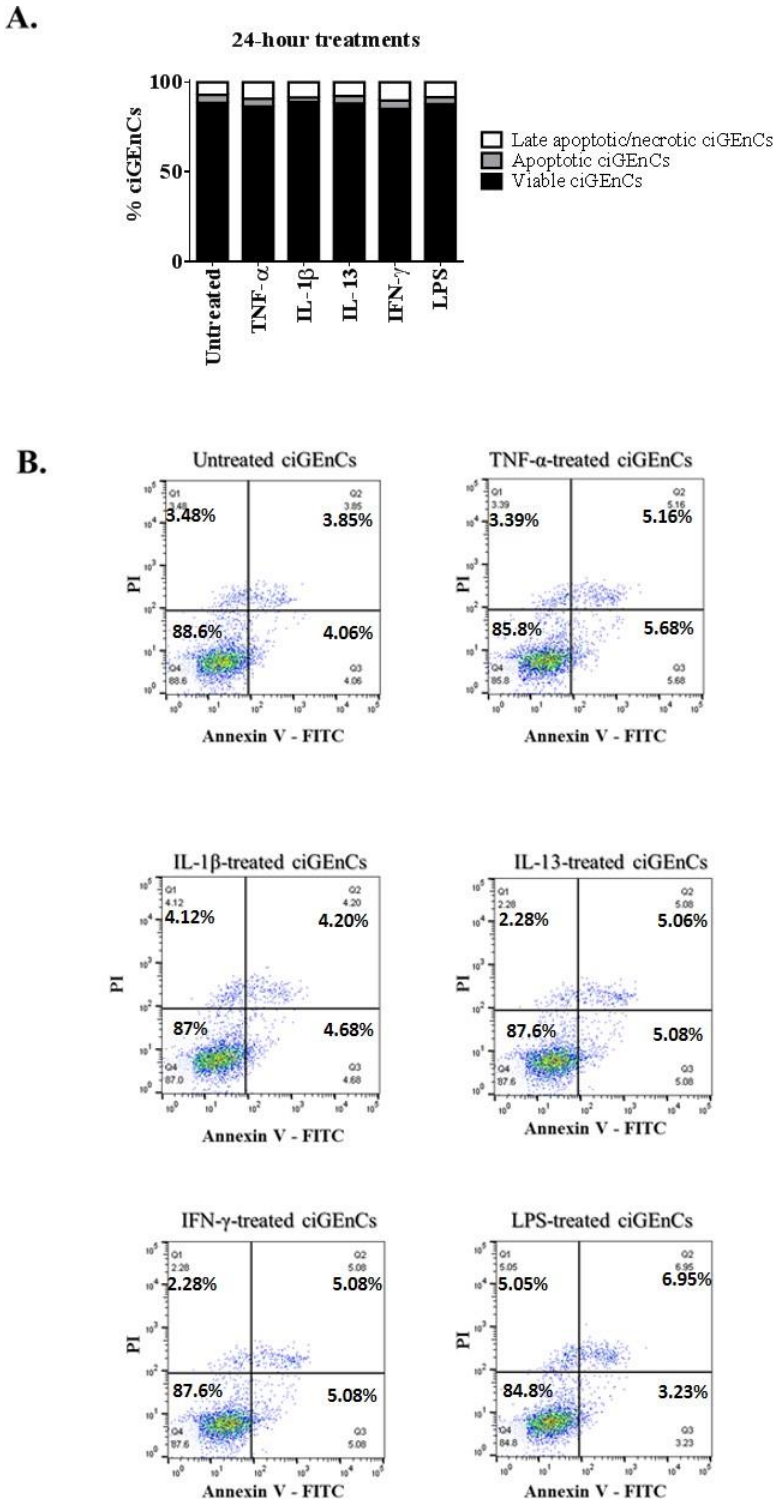


Figure 3.14 ciGEnC viability after 24 hours of TNF-α, IL-1β, IL-13, IFN-γ and LPS exposure.

A. Viable (Anx V-/PI-), early apoptotic (Anx V+/PI-) and late apoptotic/necrotic (Anx V+/PI+) ciGEnCs after 24 hours of exposure to cytokines and LPS. N=5/group. B. Representative dot-plots for each treatment.

3.4.4. TNF-α, IL-1β and LPS can induce Iκ-Bα degradation

In order to determine whether the effects observed in ciGEnCs following cytokine and LPS treatments are mediated through the NF-κB pathway, an immunoblot assay was used to determine Iκ-Bα expression. This was to evaluate its degradation levels as, under appropriate stimulation, Iκ-Bα that is bound on NF-κB in the cytoplasmic area gets degraded leading to release and subsequent NF-κB nuclear translocation[349].

At both 15 and 30 minutes of ciGEnC incubation, TNF-α and IL-1β were able to induce Iκ-Bα degradation compared to the untreated ciGEnCs whereas LPS only induced Iκ-Bα degradation at 30 minutes. At 15 minutes, TNF-α incubation led to a 2-fold decrease in Iκ-Bα levels and IL-1β led to a 3.9-fold decrease in Iκ-Bα levels (**Figure 3.15-A**). At 30 minutes, TNF-α and IL-1β induced a 1.6-fold and a 7.1-fold decrease in Iκ-Bα levels compared to the untreated ciGEnCs. In addition, LPS treatment led to a 2-fold decrease in Iκ-Bα levels (**Figure 3.15-B**). IL-13 and IFN-γ did not promote Iκ-Bα degradation (**Figures 3.15-A & 3.15-B**).

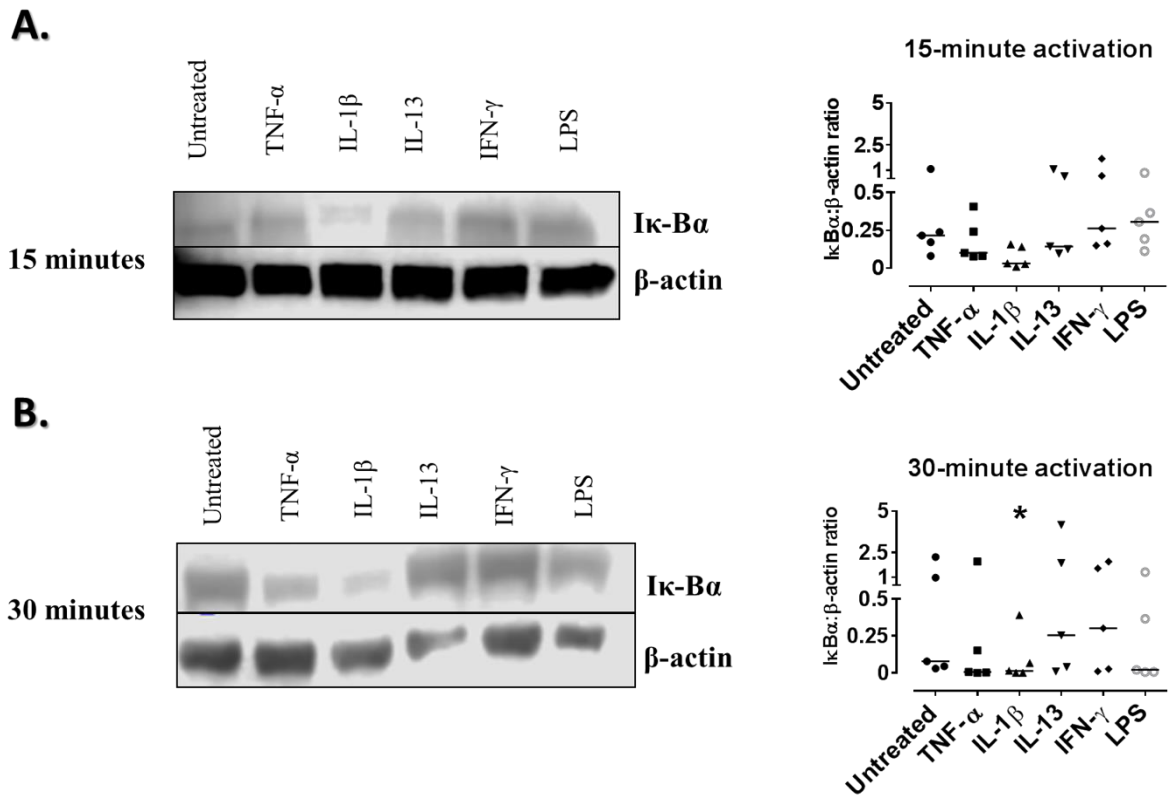


Figure 3.15 WB for Iκ-Bα protein expression after 15 and 30 minutes of ciGEnC stimulation with TNF-α, IL-1β, IL-13, IFN-γ and LPS.

*A. Representative image and graph of Iκ-Bα and β-actin Western blots, following 15-minute stimulation. B. Representative image and graph of Iκ-Bα and β-actin Western blots, following 30-minute stimulation N=5 group. Data presented as (median [range]) in ratio diagrams are analysed using Friedman test with Dunn's post-hoc test, *p≤0.05 vs untreated.*

3.4.5. Pro-inflammatory protein release is occurring through NF-κB and STAT-1 induction in ciGEnCs

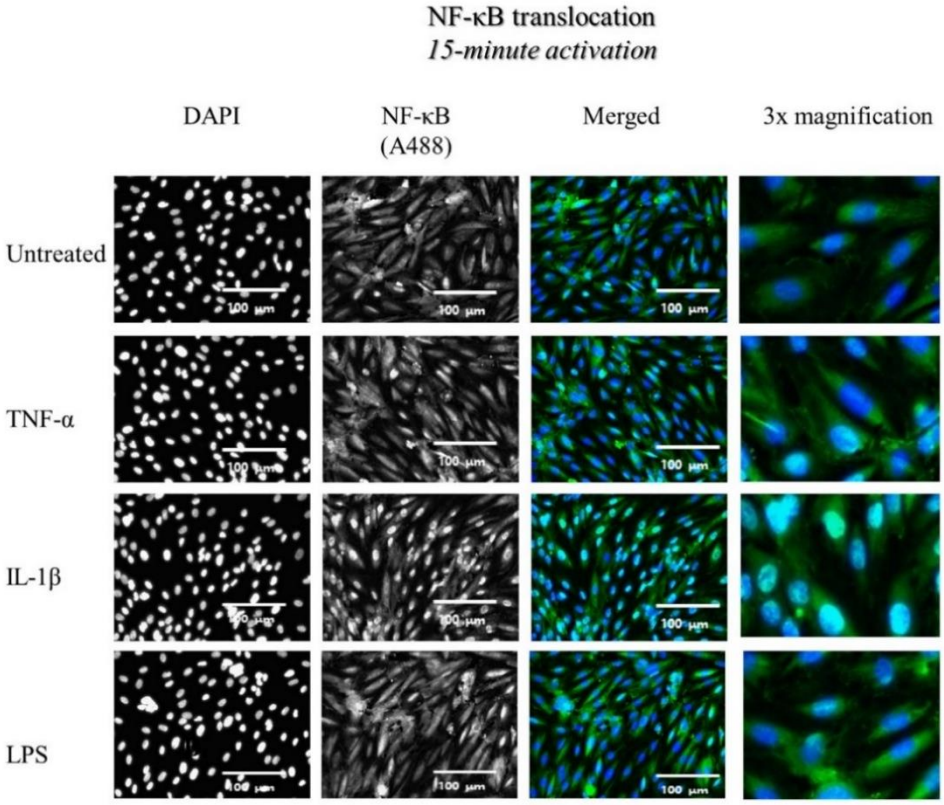
Upon activation by pro-inflammatory cytokines or LPS, pro-inflammatory transcription factors such as NF-κB, STAT-1 and STAT-2, will translocate from the cytoplasm to the nucleus of a cell leading to production of pro-inflammatory gene expression. NF-κB is activated via phosphorylation after the phosphorylation and degradation of its cytoplasmic inhibitor Iκ-Bα, whereas STATs will be activated via

phosphorylation and can subsequently form STAT-1 – STAT-1 homodimers or STAT-1 – STAT-2 heterodimers that will translocate within the nucleus.

Nuclear translocation of NF-κB following TNF-α, IL-1β and LPS stimulation and of STAT-1 and STAT-2 after IFN-γ stimulation, was used to determine pro-inflammatory pathway activation.

Based on the r-values for DAPI and A488 co-localisation, TNF-α and IL-1β promoted NF-κB nuclear translocation at both 15 (**Figure 3.16**) and 30 minutes (**Figure 3.17**) with IL-1β (**15 minutes:** r=0.7, p<0.0001, **30 minutes:** r=0.9, p<0.0001) displaying a more pronounced effect than TNF-α (**15 minutes:** r=0.4, p=0.001, **30 minutes:** r=0.7, p<0.0001) whereas LPS demonstrated a low to moderate effect only after 30 minutes (r=0.5, P=non-significant).

A.



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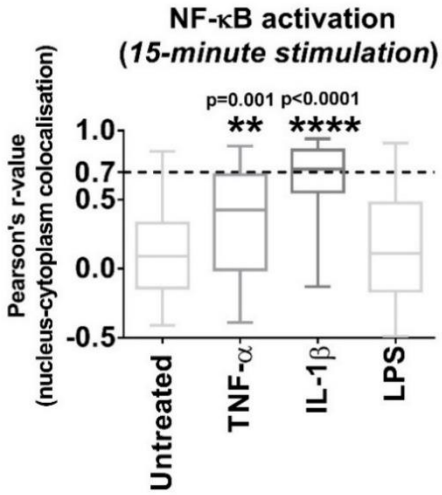
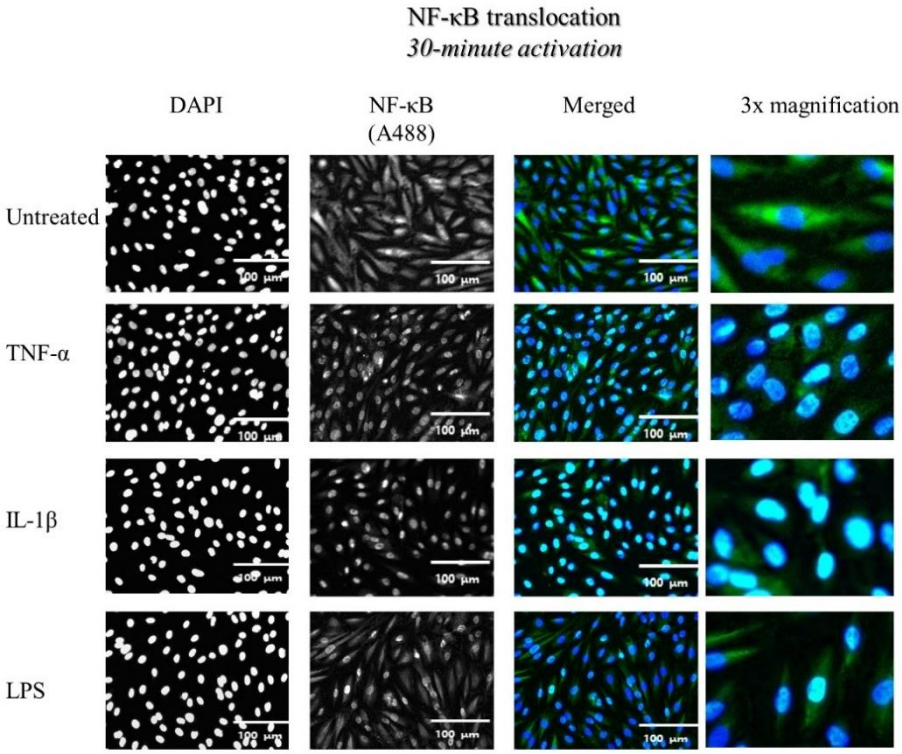


Figure 3.16 20x IF images of ciGEnCs and diagrams of statistical analysis for NF-κB activation and nuclear translocation, after 15 minutes of stimulation with cytokines and LPS.

*A. Treatments of ciGEnCs with TNF-α, IL-1β and LPS for 15 minutes for NF-κB nuclear translocation. B. Statistical analysis. Data (r-values for nuclear co-localisation of DAPI and A488 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and were analysed using Kruskal-Wallis test with Dunn's post-hoc test. **p≤0.01, ****p≤0.0001 vs untreated. Scale bars: 100µm.*

A.



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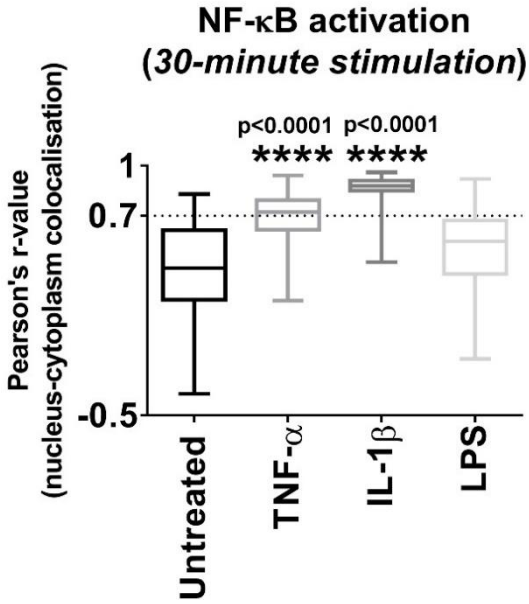

Figure 3.17 20x IF images of ciGEnCs and diagrams of statistical analysis for NF-κB activation and nuclear translocation, after 30 minutes of stimulation with cytokines and LPS.

*A. Treatments of ciGEnCs with TNF-α, IL-1β and LPS for 30 minutes for NF-κB nuclear translocation. B. Statistical analysis. Data (r-values for nuclear co-localisation of DAPI and A488 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and were analysed using Kruskal-Wallis test with Dunn's post-hoc test. ****p≤0.0001 vs untreated. Scale bars: 100µm.*

According to the r-values for DAPI and A456 co-localisation, IFN-γ moderately to highly induced STAT-1 nuclear translocation at 15 minutes ($r=0.6$, $p<0.0001$) (**Figure 3.18**) and at 30 minutes ($r=0.6$, $p<0.0001$) (**Figure 3.19**) while it could not induce STAT-2 activation (**Figures 3.20 and 3.21**).

As IL-13 altered solely VCAM-1 and not any other of the pro-inflammatory genes or proteins tested, its effect on pro-inflammatory transcription factors was not investigated.

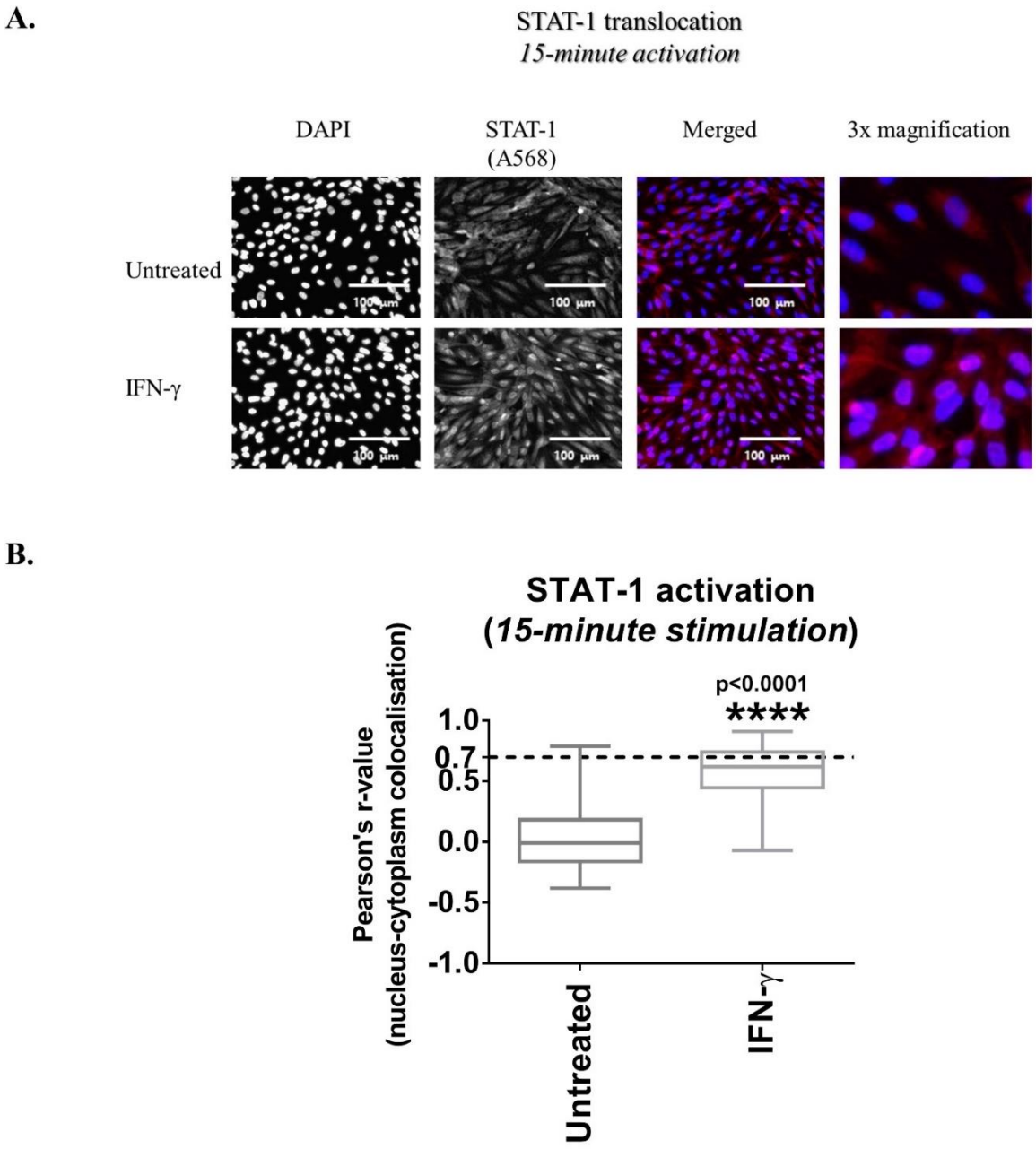

Figure 3.18 20x IF images of ciGEnCs and diagrams of statistical analysis for STAT-1 activation and nuclear translocation, after 15 minutes of stimulation with IFN-γ.

*A. Treatments of ciGEnCs with IFN-γ for 15 minutes for STAT-1 nuclear translocation. B. Statistical analysis. Data (r-values for nuclear co-localisation of DAPI and A568 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and were analysed using Mann-Whitney test. ****p≤0.0001 vs untreated. Scale bars: 100µm.*

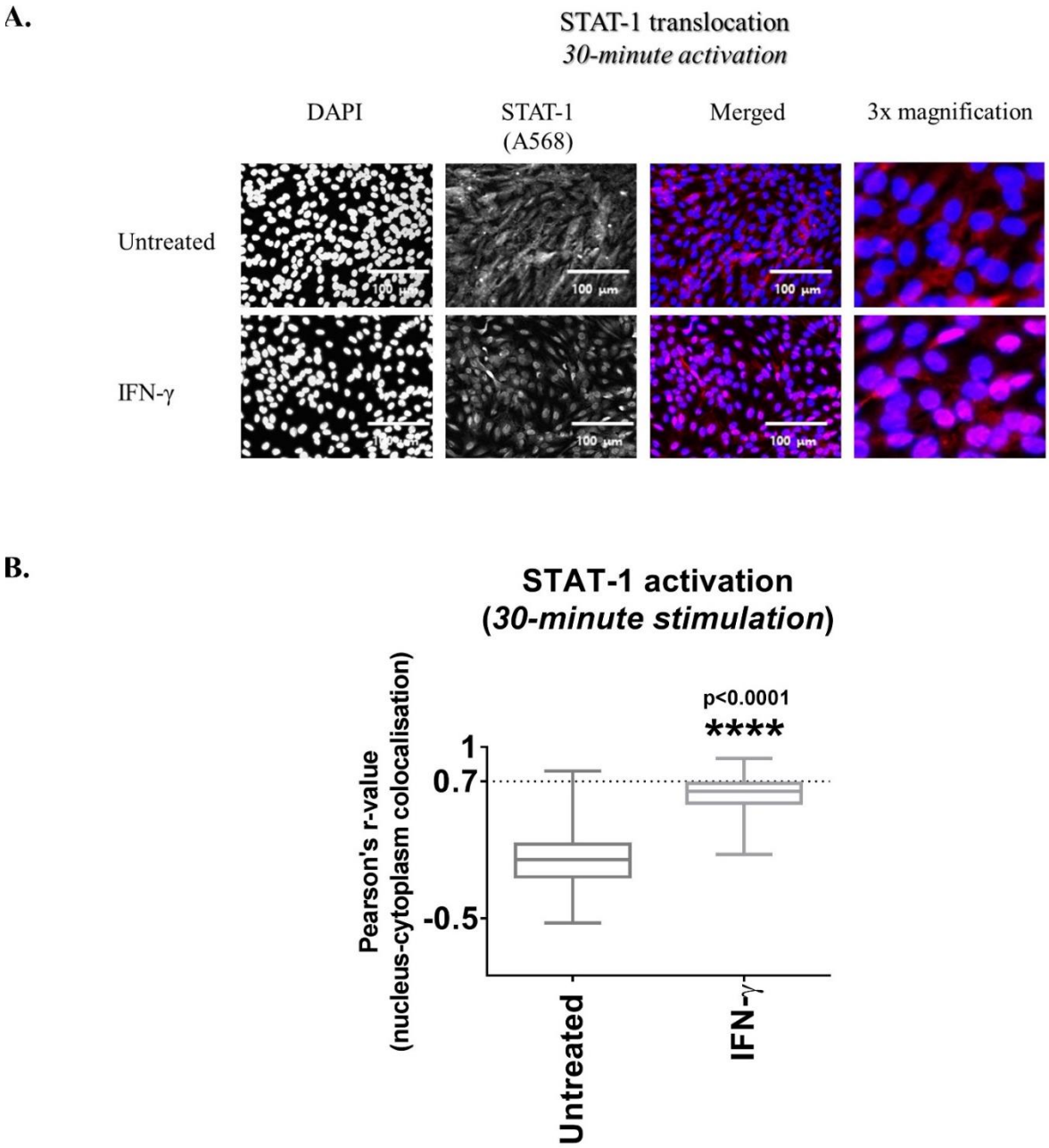

Figure 3.19 20x IF images of ciGEnCs and diagrams of statistical analysis for STAT-1 activation and nuclear translocation, after 30 minutes of stimulation with IFN-γ.

*A. Treatments of ciGEnCs with IFN-γ for 30 minutes for STAT-1 nuclear translocation. B. Statistical analysis. Data (r-values for nuclear co-localisation of DAPI and A568 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and were analysed using Mann-Whitney test. ****P≤0.0001 vs untreated. Scale bars: 100µm.*

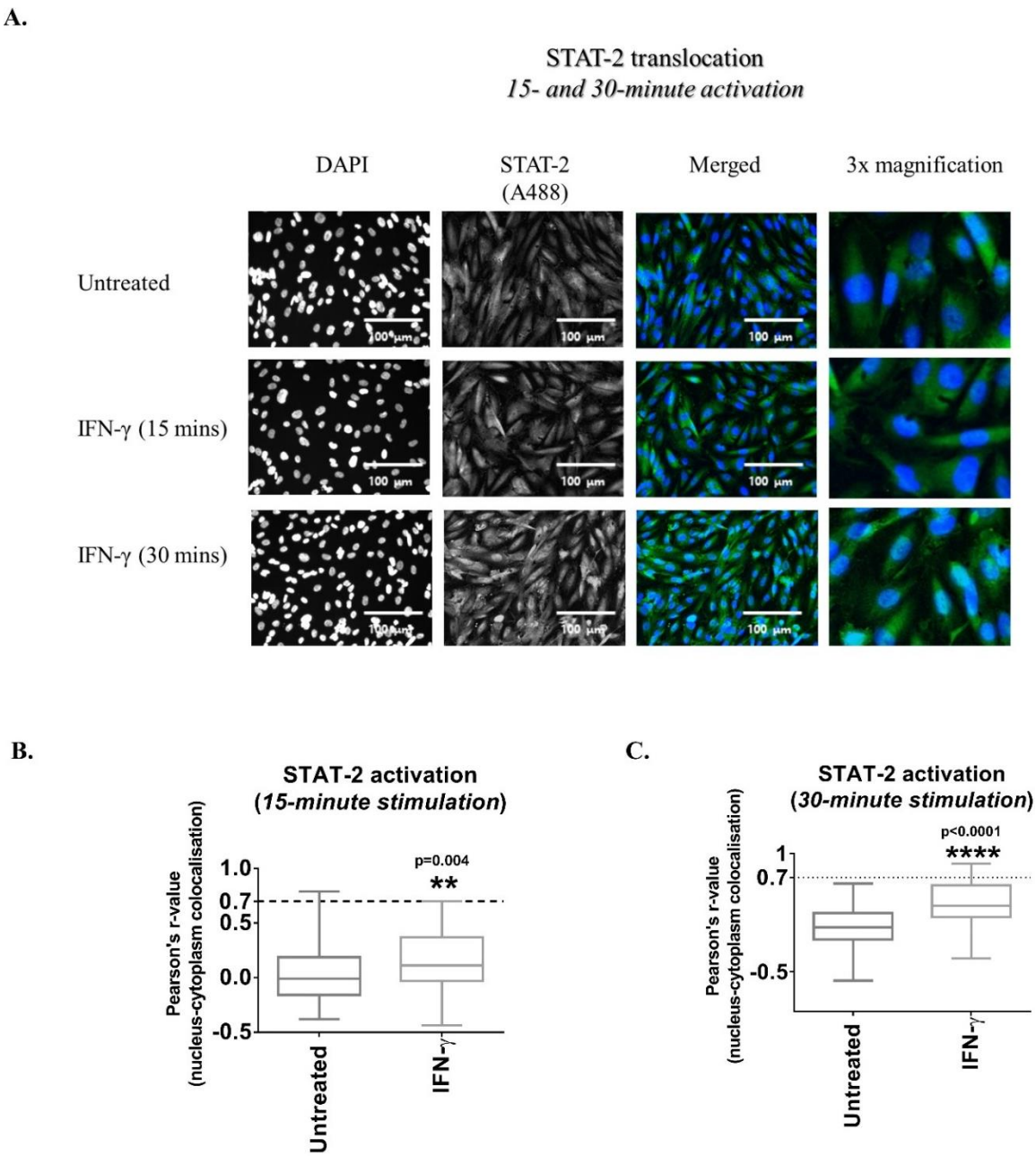

Figure 3.20 20x IF images of ciGEnCs and diagrams of statistical analysis for STAT-2 activation and nuclear translocation, after 15 and 30 minutes of stimulation with IFN-γ.

*A. Treatments of ciGEnCs with IFN-γ for 15 and 30 minutes for STAT-1 nuclear translocation. B-C. Statistical analysis. Data (r-values for nuclear co-localisation of DAPI and A488 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and were analysed using Mann-Whitney test. **p≤0.01 vs untreated. Scale bars: 100µm.*

3.5. Discussion

The experimental plan in this chapter aimed to investigate the pro-inflammatory profile of the human ciGEnCs following stimulation with cytokines known to be upregulated in the serum of patients with LN (IL-1β, TNF-α, IFN-α, IFN-γ, IL-6 and VEGF) and with LPS. In murine models of LN, LPS is known to play a critical role in LN exacerbation after a bacterial infection[350–352] but can also disrupt the normal function of human endothelial cells during human endotoxemia[353]. As physiologically the GEnCs are found at the interface between blood circulation and the glomerular renal tissue, it was hypothesised that, during LN renal disease, the GEnCs would acquire a pro-inflammatory phenotype able to enhance and promote local inflammation and, therefore, kidney-specific damage.

The ciGEnCs were treated for 15 minutes or 30 minutes and for 4 hours or 24 hours with 10 ng/ml of cytokines previously found by our laboratory and by other studies to be elevated in plasma and/or serum samples of JSLE patients with LN, or shown by other studies to be expressed in the glomerular renal tissue of JSLE or adult patients with LN, or bear certain polymorphisms associated with JSLE; IFN-α[184], IFN-γ[327,354], TNF-α[164,327], IL-1β[327,355,356], IL-6[327] and IL-13[327] and VEGF[327,357]. The potential effect of bacterial endotoxin inflammation was also examined via 1μg/ml LPS treatment, which, in murine models of LN and in human endotoxemia, is known to play a critical role in LN exacerbation after a bacterial infection[350–353].

The findings using the *in vitro* model of human ciGEnCs indicate that like HUVECs[345,358,359], HBMECs[360–362] and HMECs[363], upon activation with TNF-α, IL-1β, IL-13, IFN-γ and with LPS, ciGEnCs can increase the production of various pro-inflammatory proteins. These include cytokines (IL-6, IL-10, TNF-α, IFN-γ),

chemokines (IL-8, MCP-1, MIP-1α, IP-10), blood cell growth factors (M-CSF, GM-CSF) adhesion molecules (ICAM-1, VCAM-1, sVCAM-1) and positive co-stimulatory molecules (ICOS-L). All pro-inflammatory proteins found to be upregulated by human ciGEnCs have been previously shown to be involved in human LN or other types of human renal disease (**Table 3.1**), as described below.

Pro-inflammatory stimulus	Proteins produced by ciGEnCs
TNF-α	M-CSF, GM-CSF, MIP-1α, MCP-1, Surface VCAM-1, ICAM-1
IL-1β	IL-6, M-CSF, GM-CSF, MIP-1α, TNF-α, MCP-1, ICAM-1, ICOS-L
IL-13	Soluble and cell surface VCAM-1
IFN-γ	IL-10, IP-10, TNF-α, PD-L1
LPS	IL-6, IL-10, IP-10, MIP-1α, IFN-γ, MCP-1, ICAM-1

Table 3.1 Synopsis of proteins produced by cytokine- and LPS-mediated stimulation of ciGEnCs.

MCP-1 promotes kidney infiltration by inflammatory immune cells such as monocytes and dendritic cells in LN, and its expression within the glomerulus and can be predictive of poor renal outcomes in JSLE patients with LN[229]. MIP-1α, a chemokine which activates and attracts human granulocytes, macrophages and monocytes[364] has been shown, together with MCP-1, to promote macrophage recruitment and activation in the kidneys of patients with crescentic GN[215]. Increased mRNA levels of human *GM-CSF*, which promotes the maturation and differentiation of macrophages, neutrophils, eosinophils and basophils[205], have been found to be expressed by *in vitro* cultured human renal tubular cells derived from interstitial fibrotic kidneys compared to tubular cells

derived from non-fibrotic kidneys[313]. In addition, GM-CSF and M-CSF protein levels have been shown to be increased in human GN[213].

High levels of the pleiotropic cytokine IL-6 which -among other functions- promotes B- and T cell differentiation[171] have been shown to be produced by LN patients compared to healthy control patients[365] and high amounts of urinary IL-6 have been correlated with diffuse proliferative (class IV) active LN[173]. Serum levels of circulating TNF-α have been linked to glomerular protein expression of ICAM-1 which has been correlated with renal glomerular capillary damage in biopsy samples[161]. In addition, TNF-α gene polymorphisms leading to excessive TNF-α production can predispose patients to LN development and TNF-α protein levels have been found to be upregulated in the plasma of active LN patients[162].

IP-10 is a chemokine that mediates T cell accumulation to the inflamed tissues[190,191] and serum levels have been found to be significantly upregulated in LN patients[192] and subsequent studies have highlighted urinary IP-10 mRNA as a reliable indicator of diffuse proliferative (class IV) LN as well as a potential biomarker for treatment effectiveness[193].

Serum IL-10, which is another pleiotropic cytokine, was found to be elevated in systemic lupus erythematosus (SLE) patients with LN compared to SLE patients without LN[366] whereas plasma IL-10 has been found to be significantly elevated in JSLE patients with active disease[312]. In human LN, urinary M-CSF levels were found to be predictors of renal flares in diffuse proliferative (Class IV) LN patients[211] whereas serum and urine M-CSF could prove to be a reliable and sensitive biomarker for LN[212].

Significantly increased IFN-γ positive immune-histochemical staining has been observed in biopsies from juvenile-onset LN patients compared to healthy controls[315].

IL-8, a potent neutrophil chemokine, has been found to be one of the cytokines most commonly involved -via increased urine or serum levels- in several paediatric renal diseases, including vesicoureteral reflux[367], renal scarring[368], acute pyelonephritis[368] and idiopathic nephrotic syndrome[369].

VCAM-1 is a cell-surface protein induced upon inflammation and involved in the migration and adhesion of inflammatory cells such as lymphocytes, monocytes, eosinophils, and basophils to the vascular endothelium[254]. VCAM-1 expression as well as VCAM-1 serum and urinary levels have been shown to be significantly upregulated in LN[370,371]. Furthermore, in patients with active focal (Class III) and diffuse (Class IV) LN, the levels of sVCAM-1 were shown to be significantly elevated compared to LN patients in remission[257]. The urinary levels of ICAM-1, another adhesion molecule which is constitutively expressed by human endothelia but which is upregulated upon inflammation and promotes leukocyte binding to the endothelium[372], has been found to together with the urinary levels of VCAM-1 to be upregulated in LN patients with focal (Class III), diffuse (Class IV) and membranous (Class V) LN compared to those with minimal mesangial (Class I) and proliferative mesangial (Class II) LN and to SLE patients without renal involvement[373].

In JSLE, decreased PD-L1 expression by antigen-presenting cells has been associated with active disease[374,375]. In patients with active SLE, ICOS-L plasma levels have been found to be increased compared to patients with inactive SLE[376]. PD-L1 surface expression that was upregulated at 24 hours in ciGEnCs by IFN-γ treatment, can prevent the activation of CD-8^+ T cells[348] and inhibit the autoreactive T cell function[377]. However, after 24 hours, IL-1β significantly increased surface expression of the positive $CD4^+$ T cell co-stimulatory molecule, ICOS-L[378].

TNF-α and IL-1β, the prototypical inflammatory cytokines[379], were found to particularly upregulate MCP-1 (combined cytokine treatment led to significantly higher levels of secreted MCP-1 which could be mainly attributed to TNF-α and IL-1β combination), MIP-1α and GM-CSF. Although no statistical significance occurred with the analyses used in this study, TNF-α and IL-1β appeared to increase IL-8 secretion, as did the combined cytokine treatment. IL-1β and LPS significantly upregulated IL-6 and TNF-α secretion and LPS had a prominent effect in MIP-1α and GM-CSF secretion.

A study in which HBMECs were treated for 4, 24 and 72 hours with TNF-α and IL-1β, showed that the two cytokines differentially regulate the pro-inflammatory profile of the cells, with TNF-α inducing the highest levels of secreted IL-8, IL-6 and MCP-1 at 72 hours and IL-1β promoting higher levels of IL-8, IL-6, IP-10 and GM-CSF secretion at 24 hours, although the highest MCP-1 levels after IL-1β treatment were also observed at 72 hours[346]. In addition, HBMECs have been shown to significantly increase MIP-α secretion after stimulation with TNF-α, IL-1β and LPS for 24, 48 and 72 hours, with the highest peak being observed at 72 hours[360]. As this study only investigated the secretion of these proteins at 24 hours, further increases may be expected to occur at later time-points for IL-6, IL8, IP-10, GM-CSF and MIP-1α.

The combined cytokine treatment, which could be mainly attributed to TNF-α, IL-1β and IFN-γ led to increased amounts of secreted M-CSF. In HMECs, *M-CSF* mRNA expression has been shown to be upregulated after TNF-α treatment[363] whereas in human saphenous vein endothelial cells, TNF-α, IL-1 and LPS were found to increase *M-CSF* mRNA expression after 4 (for LPS) or 6 hours (for TNF-α and IL-1) of activation[380]. *M-CSF* mRNA in the HMECs studies was upregulated at earlier time-points than that of the 24-hour time that was used in this study; this is consistent with the hypothesis that pro-inflammatory gene transcription could occur at an earlier time-point (4 or 6 hours,

depending on the stimuli), leading to increased pro-inflammatory protein secretion at a later time-point, such as the 24 hours used in the experiments for ciGEnC M-CSF secretion.

IFN-γ predominantly increased the secretion of high amounts of IP-10 and of IL-10 as did combined cytokine treatment in which IFN-α seems to further enhance the IFN-γ effect on IP-10 and IL-10 secretion. In addition, LPS induced increased IP-10 and IL-10 secretion levels. The effect of IFN-γ on IP-10 upregulated secretion has also been observed in human brain endothelium after 48 hours[381] and therefore in ciGEnCs, further IFN-γ-mediated IP-10 increases may be expected at later time-points.

At 24 hours, IL-13 in combination with TNF-α significantly increased the surface expression of VCAM-1. This finding comes in agreement with a previous study on fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA) where elevated and sustained VCAM-1 levels were achieved by the capacity of IL-13 (or IL-4) to stabilise VCAM-1 mRNA at 24 hours of incubation, after initial stimulation with TNF-α[382]. It is important to note that FLS have been suggested to be similar to undifferentiated endothelial cells[383] and have been previously shown to share similar immune-phenotypes with HUVECs[384]. Furthermore, LPS exhibited an acute effect on increased VCAM-1 surface expression only at 4 hours but not at 24 hours. Moreover, in this study IL-13 led to a significant increase in secreted levels of sVCAM-1; IL-13 exerted an effect solely on surface and soluble VCAM-1 and not on expression or secretion of any other pro-inflammatory protein. In contrast to VCAM-1 surface expression that was cytokine-induced, ICAM-1 was constitutively expressed by the ciGEnCs and its surface expression was significantly increased by IL-1β and LPS at both 4 and 24 hours and by TNF-α only at 24 hours. ICAM-1 upregulation by TNF-α, IL-1β and LPS has also been confirmed in HUVECs in a 72-hour time-course experiment experiment[385] and therefore

further increases in ciGEnC ICAM-1 levels may be expected at a later time-point than that of 24 hours. It is important to note that the cytokines and LPS tested, had a differential effect on ciGEnC surface expression of VCAM-1 and ICAM-1, suggesting that different pro-inflammatory stimuli could lead to recruitment of different kidney-infiltrating immune cell populations.

This study mainly focused on the effect of cytokines (TNF-α, IL-1β, IFN-γ, IL-13) and LPS on inducing ciGEnC VCAM-1 and ICAM-1 surface expression. The effect of TNF-α and IL-13 on E-/P-selectin surface expression, however, was also tested. Nonetheless, basal E-/P-selectin expression by ciGEnCs was almost negligible and was not modified by TNF-α and IL-13 treatments, either alone or in combination. In contrast, IL-13 has been shown to upregulate P-selectin expression on HUVECs[386] and TNF-α has been found to upregulate expression of E-selectin by human pulmonary artery endothelial cells[387].

Other cytokines, such as IL-1β and IFN-γ and/or LPS, might have a greater effect on E-/P-selectin expression by ciGEnCs. There a strong possibility, however, the ciGEnCs might not be able to express E- and P-selectin as murine GEnCs have been shown to lack P-selectin expression[388,389]. Furthermore, limited to almost negligible levels of E- and P-selectin expression have been observed in the glomeruli of lupus-prone mice[390]. In addition, in a study using 108 human renal biopsies from patients with primary renal disease and allograft recipients and 10 healthy control renal biopsies, weak E-selectin expression in the renal vasculature was immunohistochemically detected only in two patients' renal biopsies[391]. Thus, limited expression of E- and P-selectin by human GEnCs in contrast to the more prominent expression of VCAM-1 and ICAM-1 could lead to selective recruitment of more monocytes/macrophages and lymphocytes to the inflamed glomerulus and of smaller numbers of neutrophils. It should be noted, however,

that technical and experimental limitations could have led to lack of E-/P-selectin expression of ciGEnCs, such as trypsin-induced cleavage of E- and P-selectin from the surface of ciGEnCs. For this reason, future studies could investigate the intracellular protein levels as well as the secreted protein levels and mRNA expression of E- and P-selectin by ciGEnCs. Furthermore, immunofluorescence could be used as an alternative techniqueto detect surface E- and P-selectin expression.

At 24 hours, surface levels of the constitutively expressed PD-L1 were mainly upregulated in ciGEnCs by IFN-γ treatment as has been previously shown also with HUVECs[348]. PD-L1 is known to play an important role in preventing the activation of CD-8+ T cells[348] and inhibiting the function of autoreactive T cells[377]. However, the increased presence of a variety of pro-inflammatory cytokines within the nephritic renal glomeruli could counteract the protective effect of PD-L1 by upregulating the GEnC surface expression of positive T cell co-stimulatory molecules, such as ICOS-L[378] which is preferentially expressed by the endothelium instead of the CD80 and CD86 positive co-stimulatory molecules[378]. Therefore, the balance between PD-L1 and ICOS-L, could determine the increased presence of either $CD4^+$ or $CD8^+$ T cells within the renal endothelium. Indeed, at 24 hours ICOS-L surface expression was significantly induced by IL-1β. In HUVECs, surface PD-L1 expression has been shown to be upregulated by IFN-γ alone or in combination with TNF-α after 48 hours of incubation[348] therefore suggesting further PD-L1 upregulation on ciGEnCs at later time-points after IFN-γ stimulation. Similarly to ciGEnCs, in both HUVECs and heart microvascular endothelial cells, IL-1β (but also TNF-α) was found to increase surface ICOS-L expression after a 24-hour stimulation[378].

The ciGEnC incubation time-points (4 and 24 hours) reflect an acute phase inflammation stage[284] rather than a chronic inflammation stage and future studies should

focus on longer time-response experiments, to unravel changes in expression of pro-inflammatory proteins induced by chronic exposure of ciGEnCs to pro-inflammatory stimuli. The 24-hour cytokine and LPS stimulation led to only minor levels of apoptotic/necrotic ciGEnCs, supporting the hypothesis that at this stage the human ciGEnCs maintain their integrity and acquire a pro-inflammatory phenotype due to endothelial cell activation and not due to cell death.

To further determine the effectiveness of the treatments with TNF-α, IL-1β, IL-13 and IFN-γ, concentration-response experiments were performed using serial dilutions with a 1000-fold range; the highest concentration used for all *in vitro* experimental assays was10 ng/ml and the lowest concentration was 10 pg/ml. This tested the effect of the four cytokines using concentrations similar to those that have been detected within plasma samples of patients with active or inactive LN[327]. The ciGEnCs incrementally upregulated the mRNA expression of the pro-inflammatory molecules tested in response to increasing cytokine concentrations and a positive correlation was found between increasing concentrations and mRNA levels, although not always statistically significant. Therefore, it could be speculated that as a renal flare in active LN is in progress, the presence of cytokines such as TNF-α, IL-1β, IL-13 and IFN-γ within the glomeruli will gradually increase. This increase, will lead to a local cytokine gradient formation at the areas of inflammation, affecting the surrounding ECM components, and neighbouring renal or resident/infiltrating immune cells.

Having identified the human ciGEnCs as potent pro-inflammatory contributors, the next step was to investigate which mechanistic pathways could be responsible for their activation and subsequent pro-inflammatory action. The activation of important transcription factors was explored in response to TNF-α (NF-κB), IL-1β (NF-κB), IFN-γ (STATs) and LPS (NF-κB). The transcription factor NF-κB is known to be mainly

upregulated by TNF-α and IL-1β but also by LPS, STAT-1 is known to be upregulated by IFN-γ and STAT-2 is known to be upregulated mainly by IFN-α. NF-κB, which was activated by TNF-α, IL-1β and LPS, is a central pro-inflammatory transcription factor, heavily involved in the overall pro-inflammatory response of the endothelial cells by inducing the expression of multiple downstream target genes, including; *IL-6*, *IL-8*, *IL-1β*, *MCP-1*, *MIP-1α*, *IP-10*, *GM-CSF*, *M-CSF*, *IFN-γ*, *TNF-α*, *VCAM-1* and *ICAM-1*[280,281], which were all upregulated at the 24-hour time-point following TNF-α, IL-1β or LPS stimulation.

Apart from NF-κB nuclear translocation demonstrated via IF, TNF-α-, IL-1β- and LPS-mediated NF-κB activation was further confirmed via WB for Iκ-Bα. In resting cells where no cytokine- or LPS-activation occurs, Iκ-Bα is bound to NF-κB leading to NF-κB cytoplasm retention and inhibition of its nuclear translocation[349]. However, upon cytokine- or LPS-activation Iκ-Bα is phosphorylated and degraded via the proteasome pathway, allowing for NF-κB to be phosphorylated in turn, and subsequently to be transported to the nucleus[349]. Indeed, in contrast to IFN-γ and IL-13 which did not seem to play a role in Iκ-Bα degradation, TNF-α, IL-1β and LPS did induce Iκ-Bα degradation with the 30-minute time-point exhibiting the most prominent effect on Iκ-Bα degradation for both cytokines but also for LPS. This was also confirmed via the IF experiments, where 30-minute TNF-α-, IL-1β- and LPS- activation led to increased NF-κB nuclear translocation compared to the 15-minute activation. At 15 minutes, TNF-α induced only low levels of NF-κB nuclear translocation while LPS almost failed to induce NF-κB translocation, similarly to 15-minute Iκ-Bα degradation levels. IL-1β exerted a robust effect on both Iκ-Bα degradation and NF-κB nuclear translocation at both time-points. LPS had in general the least robust effect when compared to IL-1β and TNF-α at both time-points. Indeed, a study using HUVECs to unravel their pro-

inflammatory activation pattern following IL-1β, TNF-α -and LPS stimulations after 3.75, 7, 15, 30, 60, 120 and 240 minutes found that, especially between 15 and 60 minutes of activation, IL-1β had the most robust effect in NF-κB activation and nuclear translocation compared to TNF-α and LPS, with LPS having the weakest effect in NF-κB translocation[344].

STAT-1, which was activated by IFN-γ, has been shown to induce transcriptional activation of target genes such as *IP-10, ICAM-1*, *MCP-1* and *PD-L2*[392]. In this study, IFN-γ was able to promote ciGEnCs upregulation of IP-10 and PD-L1 at 24 hours. STAT-1 activation can lead to formation of STAT-1 homodimers which subsequently translocate within the nuclei and induce the transcription of their target genes[393]. In contrast to IFN-α which also activates STAT-2 via phosphorylation leading to formation of STAT-1 – STAT-2 heterodimers[392,394], IFN-γ did not induce STAT-2 activation. Therefore, IFN-γ-mediated activation of ciGEnCs favours the formation of STAT-1 homodimers instead of STAT-1- STAT-2 heterodimers. The 30-minute IFN-γ activation seemed to have the most prominent effect in STAT-1 nuclear translocation. Regarding the role of IL-13, future studies are necessary to identify more IL-13 target genes but also to test its potential pro-fibrotic effect on ciGEnCs which could be mediated via TGF-β1 induction[395]**.** Future *in vitro* NF-κB and STAT-1 studies using knockdown assays are necessary to further verify whether the upregulated ciGEnC production of pro-inflammatory proteins is a direct cause of activation of those transcription factors.

Taken together, these data suggest that human GEnCs play a central role in LN inflammation. As demonstrated by the *in vitro* human ciGEnC model, the GEnCs are not just passive bystanders but do actively respond to the renal pro-inflammatory environment created in kidney disease, via producing a range of pro-inflammatory molecules that could exacerbate the renal inflammation. Within the nephritic kidneys,

the secretion of this variety of pro-inflammatory cytokines, chemokines, blood cell growth factors and the surface expression of adhesion molecules by the GEnCs (all summarised on **Table 3.1**) could attract and increase the numbers of glomeruli-infiltrating immune cells (**Figure 3.22**). At the same time, cytokines such as TNF-α, IL-6 or IFN-γ could activate the neighbouring podocytes and MCs (**Figure 3.22**). Therefore, the human GEnCs could be an excellent target for the development of future LN-specific therapies.

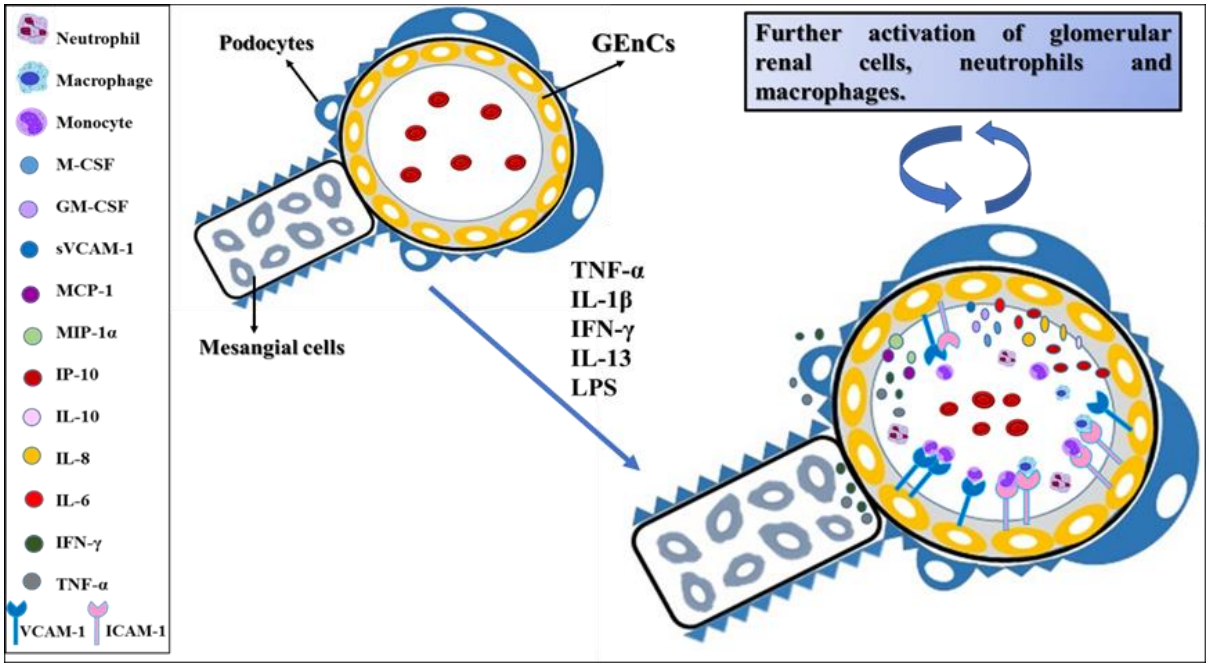

Figure 3.21 Potential effect of stimulated GEnCs in LN based on the in vitro findings.

Under the highly inflammatory environment created within the glomeruli by the presence of TNF-α, IL-1β, IL-13 and IFN-γ but also by the potential presence of LPS, the human GEnCs will be activated to produce, secrete and express a variety of pro-inflammatory cytokines, chemokines, blood cell growth factors and adhesion molecules that will further exacerbate the renal disease by either promoting the infiltration of immune cells within the glomeruli or by activating their neighbouring renal cells, the podocytes and MCs.

3.6. Conclusions

In conclusion, the human ciGEnCs were found to respond to certain pro-inflammatory cytokines and to LPS via upregulating pro-inflammatory transcription factors and producing a variety of pro-inflammatory cytokines, chemokines, blood cell growth factors, novel urinary biomarkers for active LN, adhesion and co-stimulatory molecules. The acquisition of pro-inflammatory ciGEnC phenotype is not due to cell death but due to actual ciGEnC activation.

4. Chapter 4: Investigating the effect of the renal microenvironment on the pro-inflammatory phenotype of the human GEnCs

4.1. Introduction

As demonstrated in Chapter 3, the human GEnCs are potent producers of pro-inflammatory proteins, actively contributing to the renal pro-inflammatory environment in LN. More specifically, using an *in vitro* model of LN, the human ciGEnCs were found to produce a variety of proteins: adhesion molecules able to mediate leukocyte binding (VCAM-1, ICAM-1), positive T cell co-stimulatory molecules (ICOS-L) and cytokines (TNF-α, IFN-γ, IL-6, IL-10), chemokines (IL-8, IP-10, MIP-1α) and blood cell growth factors (M-CSF, GM-CSF) that could promote leukocyte infiltration, maturation and activation within the kidneys. Furthermore, the cytokines produced by the GEnCs could potentially activate their neighbouring glomerular cells; podocytes and MCs.

However, by directly interacting with the blood circulating factors and with rest of the glomerular renal cells, the human GEnCs may not only affect their surrounding environment but, in turn, they may also be affected by this environment. The presence of pro-inflammatory cytokines within the glomerulus, some of them produced by the GEnCs themselves, could lead to podocyte and mesangial cell activation. Thus, the production of more pro-inflammatory cytokines could in turn further activate the GEnCs. Furthermore, infiltrating immune cells such as neutrophils and macrophages are among the first to reach the sites of inflammation early at the acute inflammatory phase, with neutrophil recruitment preceding that of macrophages[396,397]. Thus, these circulating immune cells could further enhance GEnC activation via release of a variety of mediators. Pro-inflammatory mediators released by all of the different types of immune cells (innate and adaptive immune system-derived), could also affect the GEnCs via their

presence in the blood plasma/serum, leading to expression of pro-inflammatory genes and of genes associated with ECM production and remodelling that are involved in renal tissue fibrosis and CKD[398,399].

Exploring the effect of the renal microenvironment on the GEnCs will help gain insight into the complex interactions between GEnCs and the rest of the cell types that communicate with the glomerular endothelium. Therefore, a more physiological *in vitro* model of LN could be developed, imitating the interactions taking place within the kidneys of the human organism.

4.2. Aim

To investigate the potential effect of the glomerular renal microenvironment on GEnC pro-inflammatory profile using multiple *in vitro* ciGEnC models.

4.2.1. Objectives

Objective 1: To assess whether ciGEnC treatments with JSLE patient and paediatric healthy control sera can modify their fibrotic and pro-inflammatory phenotype.

To address this objective, sera from JSLE patients with active and inactive LN and sera from healthy controls were used to stimulate ciGEnCs *in vitro*. Subsequently, changes in the expression of fibrosis-related genes and of pro-inflammatory genes (same as those tested in gene and protein level in Chapter 3), were investigated.

O**bjective 2:** To determine whether glomerulus-infiltrating immune cells such neutrophils and macrophages could potentially have a paracrine effect the pro-inflammatory activation of GEnCs.

To address this objective, conditioned media from stimulated human neutrophils, macrophages and THP-1 cells were used to *in vitro* stimulate the ciGEnCs; subsequently potential changes in the expression of pro-inflammatory genes/proteins were tested.

O**bjective 3:** To determine whether GEnC-neighbouring renal cells, podocytes and MCs, could potentially have a paracrine effect the pro-inflammatory activation of GEnCs.

To address this objective, conditioned media from stimulated conditionally immortalised human podocytes and MCs were used to *in vitro* stimulate the ciGEnCs; subsequently potential changes in the expression of pro-inflammatory genes were tested.

4.2.2. Chapter hypothesis

The GEnCs do not only affect their microenvironment via producing pro-inflammatory factors upon renal disease but they can also be affected by their microenvironment in return. Thus, *in vitro* ciGEnC treatment with serum samples from patients with active LN will upregulate the production of pro-inflammatory genes and genes related to ECM production/remodelling, compared to serum samples from patients with inactive LN and HCs. Furthermore, ciGEnC treatment with conditioned media from cytokine- and LPS-activated neutrophils, macrophages, podocytes and MCs, will promote the expression of pro-inflammatory genes and production of pro-inflammatory proteins.

4.3. Materials and Methods

4.3.1. ciGEnC preparation and treatment

Fully differentiated ciGEnCs were seeded on 12-well plates for all treatments. Subsequently, they were incubated at 37^{o}C (with 5% CO_2) for 24 hours (h) with serum

samples or with conditioned media of previously stimulated neutrophils, macrophages, and podocytes or MCs. A minimum of three consecutive passages of ciGEnCs was used for all experiments.

4.3.1.1.Serum treatments

The ciGEnCs were treated for 4 or 24 hours with 5% sera in media without FBS from JSLE patients with active LN (renal BILAG A/B) and inactive LN (renal BILAG D/E) and from healthy controls (HCs; n=10/group). For these experiments, the age and sex of healthy controls was matched to those of JSLE patients whenever that was possible. The median age of HCs was 13 years, the median age of active LN patients wa 15 years and the median age of inactive LN patients was 14.5 years; 90% of active and inactive LN patients and 70% of HCs were females. Patients' and HCs' demographic information is presented on **Table 4.1.** Following stimulation, the cells were washed in PBS and lysed in Trizol for RNA extraction.

Demographics	Active LN (10)	Inactive LN (10)	Healthy Controls (10)
Age (years) (median [range])	15 [9-17]	14.5 [11-18]	13 [9-17.2]
Age at diagnosis (years) (median [range])	12.4 [6.28-14.48]	11.73 [9.3-15.59]	-
Females (%)	90 (9)	90 (9)	70 (7)
Nationality (%)			
White British	0	0	90 (9)
Black British	10 (1)	0	0
British	10 (1)	10 (1)	0
Indian	0	30 (3)	0
Nepalese	0	10 (1)	0
Pakistani	10 (1)	30 (3)	0
Bangladeshi	10 (1)	0	0
Chinese	10 (1)	0	0
African	40 (4)	10 (1)	0
Caucasian	0	10 (1)	0
Not stated	10 (1)	0	10 (1)
Renal BILAG score (%)			-
A	30 % (3)		
B	70 % (5)		
D		80 % (8)	
E		20 % (2)	
Renal disease manifestations (%)			
Proteinuria (ACR>100mg/mmol)	70 (7)	-	
Nephrotic Syndrome	10 (1)	-	
Renal Hypertension	10 (1)	-	
Low GFR (<80 ml/min/1.73m^2)	10 (1)	-	-
Increased plasma creatinine (>130 µmol/ml)	10 (1)	-	
Urine ACR (mg/dL) (median [range])	10 (1)	-	
Previous renal involvement	-	80 (8)	
Biopsy-proven LN (%)	70 (7)	30 (3)	-
LN class (WHO or ISN/RPS) (%)			
II	10 (1)	-	
III/IV	10 (1)	-	
IV	-	30 (3)	
IV-C	10 (1)	-	
V	20 (2)	-	
IV/V	10 (1)	-	
Mixed	10 (1)	-	
Medications dosage [range] (patient n-number)			
Hydroxychloroquine	200 mg [200-700] (7)	200 mg [200-300] (7)	
Azathioprine	150 mg [100-300] (3)	100 mg [100-100] (2)	
Mycophenolate mofetil	1500 mg [1200-2000]	1500 mg [750-2000]	
Prednisolone	(5)	(6)	
Methotrexate (oral)	7.5 mg [2.5-45] (6)	10 mg [2-10] (6)	-
Rituximab	-	20 mg [17.5-22.5] (2)	
Cyclophosphamide	2000 mg, 6 pulses (1)	-	
IVIG	520 mg/m^2 (1)	-	
	-	2 g (1)	

Table 4.1 Patient and healthy control demographics.

4.3.1.2.Neutrophil conditioned media treatments

Neutrophils isolated from healthy volunteers (see section 2.2.1) (n=5) were re-suspended in RPMI (+10% FBS), seeded onto 12-well plates at a concentration of

$1x10^6$/mL and stimulated for 1h with 10ng/mL TNF-α, 10ng/mL IFN-γ or 1μg/mL LPS. Following this, neutrophils were collected and centrifuged at 200*xg* for 10 mins to pellet the cells and the conditioned media was collected and stored at -80°C until use.

The neutrophil conditioned media was used to treat fully differentiated ciGEnCs for 24h. Following stimulation, the cells were washed in PBS and lysed in Trizol for RNA extraction.

4.3.1.3.Primary macrophage conditioned media treatments

PBMCs were isolated from healthy volunteers (see section 2.2.1) (n=7) and were re-suspended in RPMI (+10% FBS). These were seeded into 12-well plates at 37°C for 2h, following this the non-adherent lymphocytes were removed, and the adherent monocytes were treated with RPMI (+10% FBS, 1% penicillin-streptomycin and 50ng/mL M-CSF) to aid their differentiation into macrophages. These were then incubated at 37°C for 7 days. Macrophage differentiation was confirmed using light microscopy (**Figure 4.1**).

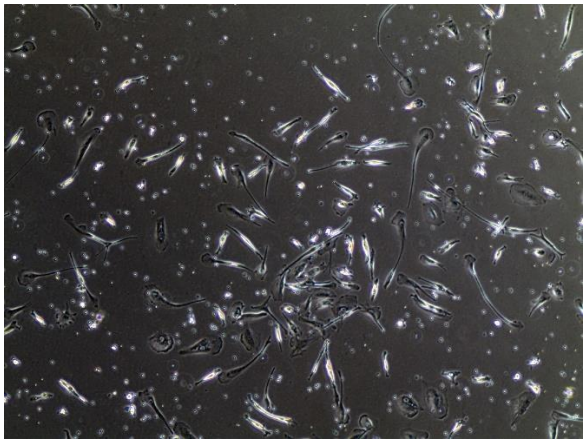

Figure 4.1 Macrophages following 7-day treatment with 50ng/mL M-CSF.

Macrophages were then treated for 48 hours with 10ng/mL IFN-γ or 1μg/mL LPS at 37°C, following this the conditioned media were collected and stored at -80°C until use.

The macrophage conditioned media were used to treat fully differentiated ciGEnCs for 24h. Following stimulation, the cells were washed in PBS and lysed in Trizol for RNA extraction.

4.3.1.4.THP-1 cell line conditioned media treatments

Primary human macrophage cultures yielded low numbers of cells due to the small volumes of blood (<20mL) permitted by our ethics. Thus THP-1 macrophages were used to further explore the paracrine effects of human macrophages on GEnCs.

Undifferentiated THP-1 cells in suspension were seeded into 6 well plates at a concentration of 750,000 cells per well. The THP-1 cells were then treated with 100 ng/ml of PMA in RMPI (+ 10% FBS) at 37ºC (5% CO_2) for 4-5 days to aid their differentiation into fibroblast-like macrophages and their attachment to the bottom of the plates (**Figure 4.2**).

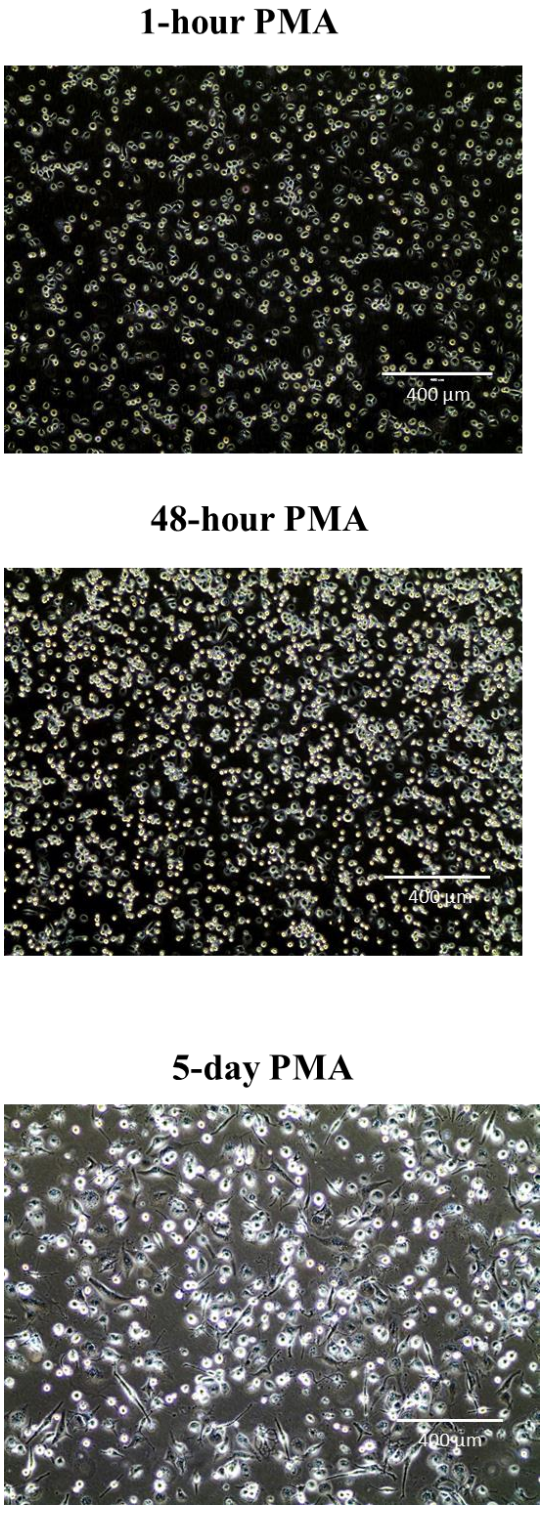

Figure 4.2 THP-1 macrophage culture.

Before PMA stimulation, the undifferentiated THP-1 cells are non-adherent and have a spherical shape. At 48 hours after PMA stimulation the THP-1 cells attach to the bottom of the plates and start changing their morphology by developing some protrusions. By the end of the 5-day incubation with PMA, the differentiated THP-1 macrophages attain a fibroblast-like morphology.

Following differentiation, the THP-1 macrophages were treated with fresh media for 24h and then incubated with 10ng/mL IFN-γ or 1μg/mL LPS in RPMI (+10% FBS) for 48h. Conditioned media was collected and frozen at -80°C until use.

TNF-α and IL-1β secretion into the conditioned media was assessed using R&D DuoSet ELISA analysis as described in section 2.2.5. The conditioned media was then used to treat the ciGEnCs for 24h. Additionally conditioned media were treated for 2h with 1μg/mL anti-human TNF-α Ab and with 10μg/mL human recombinant interleukin-1 receptor antagonist (IL-1Ra), following treatment this conditioned media was used to treat ciGEnCs for 24h to determine the effects of TNF-α and IL-1β blockade. Following stimulation, the ciGEnCs were washed in PBS and lysed in Trizol for RNA extraction.

4.3.1.5.Podocyte and mesangial cell conditioned media treatments

Conditionally immortalised human podocytes[259] and MCs[400] kindly provided by Professor Moin Saleem (Children's Renal Unit and Academic Renal Unit, University of Bristol, UK) were initially cultured at 33°C (5% CO_2) in RPMI-1640 (+L-glutamine) with 10% FBS and 1% ITS. Upon reaching 50-70% confluence, podocytes and MCs were seeded into 12-well plates and transferred at 37 °C (5% CO_2) for 10-14 days and 7-10 days respectively, until they were fully differentiated. The fully differentiated podocytes and MCs were treated for 24 hours with 10 ng/ml of key cytokines involved in LN and JSLE pathogenesis - TNF-α, IL-1β, IFN-α, IFN-γ - and with 1 μg/ml of LPS.

Subsequently, the podocyte and MC conditioned media were collected and were used to treat the ciGEnCs for 24 hours. The ciGEnCs were then washed with PBS and lysed in Trizol for RNA extraction.

4.3.2. qRT-PCR analysis

RNA was extracted and transcribed to cDNA as previously described in the Materials and Methods (Section 2.2.3). This cDNA was used for qRT-PCR analysis to investigate changes in mRNA expression levels for genes listed in **Table 4.2**.

Gene	Forward Primer Sequence (5'- 3')	Reverse Primer Sequence (5'- 3')
ACTB	CATTGCGGTGGACGATGGA	AGATCAAGATCATTGCTCCTCCTG
TBP	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
TUBB	GGACCGCATCTCTGTGTACT	CTGCCCCAGACTGACCAAATA
YWHAZ	ACTGGGTCTGGCCCTTAACT	GGGTATCCGATGTCCACAATGTC
TNF-α	CTTCTGCCTGCTGCACTTTG	GGGTTTGCTACAACATGGGC
IL-1β	CAACAGGCTGCTCTGGGATT	CCTGGAAGGAGCACTTCATCT
IL-6	AGAGGCACTGGCAGAAAACA	TCACCAGGCAAGTCTCCTCA
IL-8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA
IL-10	GCGCTGTCATCGATTTCTTCC	GCCACCCTGATGTCTCAGTT
MIP-1α	CATTCCGTCACCTGCTCAGA	AGCAGCAAGTGATGCAGAGA
IP-10	AGCCCCACGTTTTCTGAGAC	GAGAGAGGTACTCCTTGAATGCC
M-CSF	CCCCAGTCCTCTCTTAAAAGGC	GAGAGGACCCAGGCAAACTT
GM-CSF	GAGACACTGCTGCTGAGATGAA	AGGAAGTTTCCGGGGTTGGAG
MCP-1	CTCGCTCAGCCAGATGCAAT	TCTCCTTGGCCACAATGGTC
VCAM-1	GGTGGGACACAAATAAGGGT	GCTTGAGAAGCTGCAAAC
TF	TTCACTGGCCCAAGTGGTTT	AGAAGGAAGGGCTCAGTTGC
L-PGDS	CCAGGGCTGAGTTAAAGGAGA	CCCTGGGGAGTCCTATTGTTC
TNFR1	CTGGAGCTGTTGGTGGGAAT	TTGTGGCACTTGGTACAGCA
TNFR2	CACCGGGAGCTCAGATTCTTC	CACTGTGAGCTGTGGTCAGA
IL-1RI	GCGGCAGGAATGTGACAA	TACCAAGGGGTCCAGCTTCT
IL-1RII	TGAGCAGCAACAAGGCCA	CAACACGTACAAGCGCAACA
COL1A1	CCACGCATGAGCGGACCCTAA	ATTGGTGGGATGTCTTCGTCTTGG
COL1A2	ACAAGGCATTCGTGGCGATA	ACCATGGTGACCAGCGATAC
COL3A1	GACCTGGAGAGCGAGGATTG	GTCCATCGAAGCCTCTGTGT
COL4A1	GCCAGCAAGGTGTTACAGGATT	AGAAGGACACTGTGGGTCATCTATT
LAMB1	CCGGAAAGGAAGACGGGAAG	CGCCAGGTCCTGCTGTTTCTAA
LAMB2	CAGGCAGAGTTGACACGGAA	AGCCAGCACGCTTAGCAGTAG
TIMP1	GGAATGCACAGTGTTTCCCT	GCCCTTTTCAGAGCCTTGGA
MMP2	CCATGAAGCCCTGTTCACCA	CTTCTTGTCGCGGTCGTAGT
MMP7	GGGAACAGGCTCAGGACTAT	CTCCACATCTGGGCTTCTGC
MMP9	GGCGCTCATGTACCCTATGT	TTCAGGGCGAGGACCATAGA
FN	TCGCAGCTTCGAGATCAGTG	CCCCTCTTCATGACGCTTGT

Table 4.2 Primers used for qRT-PCR.

4.3.3. Statistical Analyses

Changes between two different groups were studied using non-parametric Mann-Whitney test whereas changes among three or more groups were studied using Kruskal-

Wallis or Friedman's test with Dunn's post-hoc test, using the GraphPad Prism 4.0 software. For changes after neutrophil, conditionally immortalised podocyte and mesangial cell conditioned media, multiple t-tests were performed with p-value corrections for multiple comparisons using the Holm-Sidak method. For changes after THP-1 conditioned media (with or without anti-TNF-α/IL-1Ra addition), Kruskal-Wallis test was performed with corrected p-values.

4.3.4. TNF-α blocking assay using anti-TNF-α optimisation

To verify that anti-TNF-α and IL-1Ra target and inhibit the pro-inflammatory effects of TNF-α and IL-1β respectively, ciGEnCs were seeded onto 12-well plates and following differentiation were treated for 24h with 10 ng/ml of TNF-α alone or in combination with 1 μg/ml of anti-TNF-αAb and with 10 ng/ml of IL-1β alone or in combination with 1 μg/ml or 10 μg/ml of IL-1Ra. After the 24h treatment, the ciGEnC conditioned media were collected and used for Luminex ELISA, while the ciGEnCs were lysed with Trizol for RNA extraction. In addition, treated ciGEnCs were assessed for the expression of NF-κB using IF staining.

TNF-α can elicit its effects on GEnCs through activation and translocation of NF-κB to the nucleus, therefore NF-κB nuclear localisation was assessed in untreated cells where the majority of the transcription factor was found in the cytosol as demonstrated by low co-localisation of DAPI and NF-κB staining (r-value: 0.02 [-0.4-0.92])). Upon treatment with 10ng/mL TNF-α for 30 mins NF-κB staining co-localised with DAPI staining suggesting nuclear translocation was occurring (r-value: 0.73[-0.15-0.95). Following pre-treatment with 1μg/mL anti-TNF-α nuclear levels of NF-κB (r-value: 0.24 [-0.61-0.91]) returned similar to those seen in untreated cells (r-value: 0.02 [-0.4-0.92])) (**Figure 4.3**).

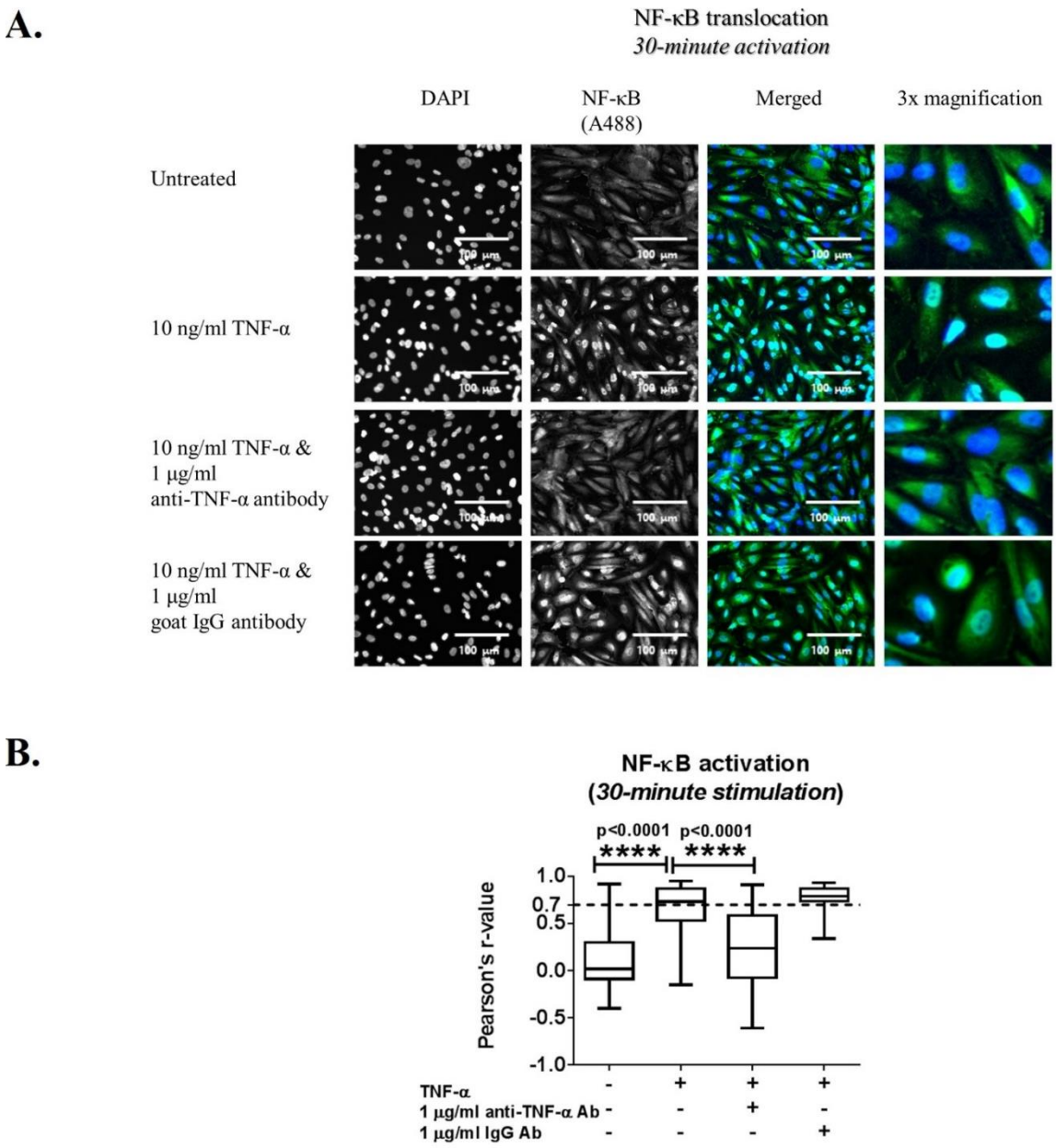

Figure 4.3 Effect of anti-TNF-αAb treatments on TNF-α-mediated NF-κB nuclear translocation.

*A. 20x IF images of ciGEnCs following 30-minute stimulation with TNF-α alone or with TNF-α together with 1 μg/ml of anti-TNF-αAb or 1 μg/ml of goat IgG B. Statistical analysis diagram. Data (r-values for nuclear co-localisation of DAPI and A488 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and are analysed using Mann-Whitney test. ****p≤0.0001 vs TNF-α. Scale bars: 100μm.*

After 24 hours of combined treatment with 10 ng/ml of TNF-α and with 1 μg/ml of anti-TNF-αAb, the ciGEnCs displayed similar levels of *IL-8*, *GM-CSF*, *MCP-1*, *MIP-1α* and *VCAM-1* mRNA expression, compared to those of the untreated ciGEnCs (**Figure 4.4**). Changes in the mRNA expression of these genes were tested as in Chapter 3 their expression was shown to be affected in a TNF-α concentration-dependent manner (section 3.4.3, Figure 3.1).

More specifically, *GM-CSF* mRNA levels of untreated ciGEnCs (0.001 [0.001-0.002], P=0.02) were significantly lower than those of TNF-α-treated cells (0.03 [0.004-0.05]) whereas a trend was observed for lower *GM-CSF* mRNA levels after anti-TNF-α treatment (0.002 [0.0008-0.003], P=0.06) compared to TNF-α-treated ciGEnCs (**Figure 4.4-A**). Similarly, *IL-8* mRNA levels dropped close to untreated ciGEnCs' baseline levels (0.04 [0.025-0.07], P=0.04) and a trend was observed after anti-TNF-α treatment (0.05 [0.02-0.12], p=0.079) compared to TNF-α-treated ciGEnCs (0.5 [0.08-0.86]) (**Figure 4.4-B**).

MCP-1 mRNA levels were significantly decreased after anti-TNF-α treatment (0.1 [0.04-0,4], P=0.02) and reached similar levels to those of untreated ciGEnCs (0.07 [0.06-0.11], P=0.006) compared to TNF-α-treated cells (1.15 [0.15-2.9]) (**Figure 4.4-C)**. Although no statistically significant changes occurred in *VCAM-1* mRNA expression of untreated and anti-TNF-α-treated ciGEnCs compared to TNF-α-treated ciGEnCs, the *VCAM-1* mRNA levels were considerably lower than those of TNF-α-treated ciGEnCs (**Figure 4.4-D)**.

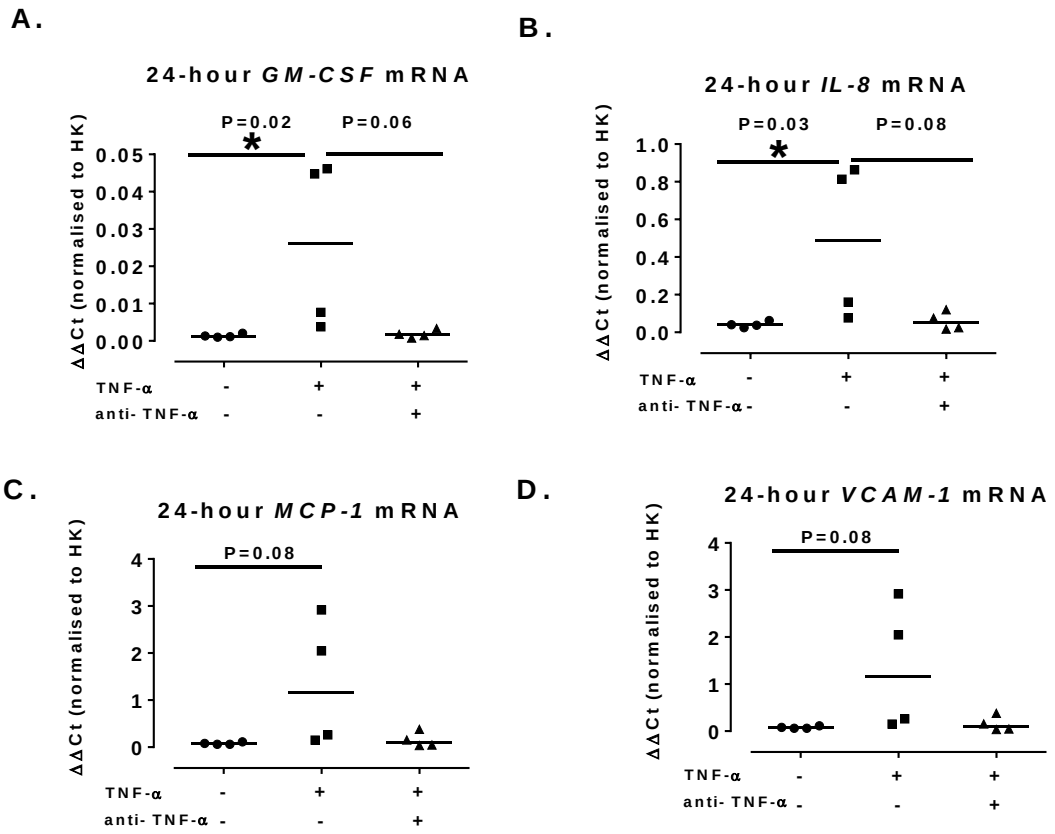

Figure 4.4 Effect of anti-TNF-α Ab treatments on TNF-α-mediated pro-inflammatory gene expression.

*Changes in the mRNA expression levels of GM-CSF (A), IL-8 (B), MCP-1 (C), and VCAM-1 (D) following 24-hour treatments with TNF-α alone or with TNF-α together with 1 μg/ml of anti-TNF-α Ab. N=4/group. Data presented as median [range] are analysed using Kruskal-Wallis test, with Dunn's post-hoc test, *P≤0.05 vs untreated. HK: ACTB, TUBB, TBP.*

Similarly, GM-CSF, IL-8, MIP-1α and M-CSF secreted protein levels of ciGEnCs treated with (TNF-α + anti-TNF-α Ab), were more similar to those of untreated ciGEnCs compared to those of TNF-α-treated ciGEnCs (**Figure 4.5**). More specifically, TNF-α-mediated secretion was statistically significantly increased (174pg/ml [41-354], p=0.03) compared to untreated ciGEnCs (14pg/ml [12-18]) and anti-TNF-α antibody (Ab)

addition (33pg/ml [12-56]) brought GM-CSF levels closer to those of untreated ciGEnCs (**Figure 4.5-A**).

Although not statistically significantly higher, IL-8 levels of TNF-α-treated ciGEnCs (4,450pg/ml [2,791-5,740]) were more elevated compared to those of untreated [1,197pg/ml [988-2,004]) and (TNF-α + anti-TNF-α Ab)-treated ciGEnCs [2,636pg/ml [843-5,331]) (Figure 4.5-B). Similarly, the MIP-1α levels of TNF-α-treated ciGEnCs (4,450pg/ml [2,791-5,740]) were higher -but not statistically significantly- compared to those of untreated [1,197pg/ml [988-2,004]) and (TNF-α + anti-TNF-α Ab)-treated ciGEnCs [2,636pg/ml [843-5,331] (**Figure 4.5-C**).

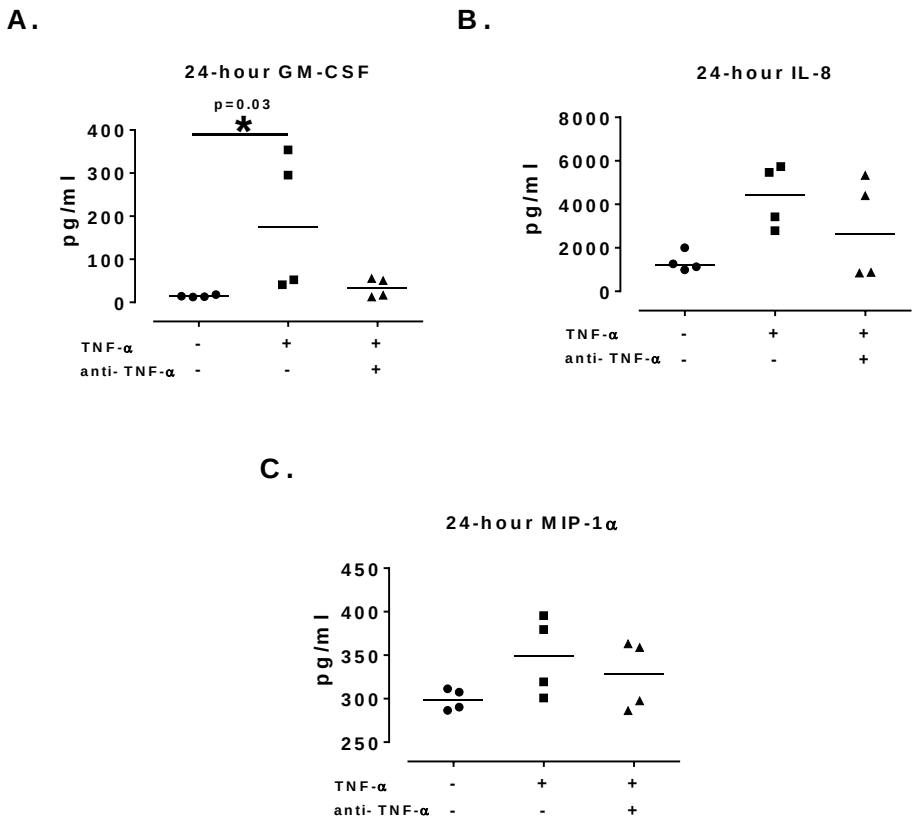

Figure 4.5 Effect of anti-TNF-αAb treatments on TNF-α-mediated pro-inflammatory protein secretion.

*Changes in the secretion levels of GM-CSF (A), IL-8 (B), MIP-1α (C) following 24-hour treatments with TNF-α alone or with TNF-α together with 1 μg/ml of anti-TNF-α Ab. N=4/group. Data presented as median [range] are analysed using Kruskal-Wallis test, with Dunn's post-hoc test, *P≤0.05 vs untreated.*

4.3.5. IL-1β blocking assay using IL-1Ra optimisation

Similar to TNF-α, IL-1β can also elicit its effects on GEnCs through activation and translocation of NF-κB to the nucleus, therefore NF-κB nuclear localisation was also assessed in untreated cells; in untreated cells the majority of the transcription factor was found in the cytosol as demonstrated by low co-localisation of DAPI and NF-κB staining (r-value: 0.31 [-0.32-0.93]).

After IL-1β inhibition with IL-1Ra, IF assay revealed that the nuclear translocation of NF-κB was moderately suppressed with 1 μg/ml of IL-1Ra (r-value: 0.74 [-0.26-0.96]) and more strongly suppressed with 10 μg/ml IL-1Ra (r-value: 0.52 [-0.25-0.9]) compared to IL-1β-treated ciGEnCs (r-value: 0.85 [-0.06-0.97]) (**Figure 4.6**). The untreated ciGEnCs displayed the lowest levels of NF-κB activation (r-value: 0.31 [-0.32-0.93]) (**Figure 4.6**).

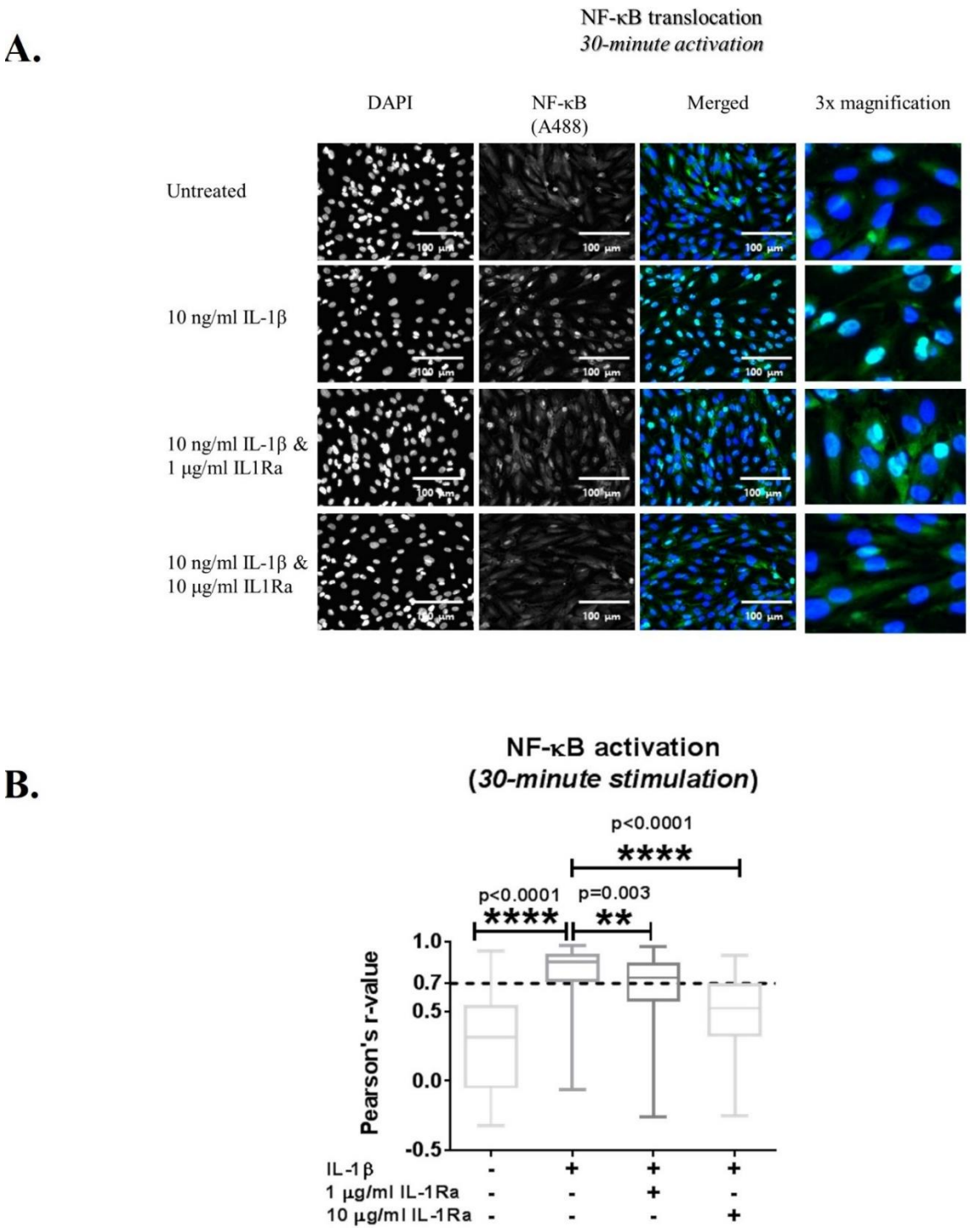

Figure 4.6 Effect of IL-1Ra treatments on IL-1β-mediated NF-κB nuclear translocation.

*A. 20x IF images of ciGEnCs following 30-minute stimulation with IL-1β alone or with IL-1β together with 1 or 10 μg/ml IL-1Ra. B. Statistical analysis diagram. Data (r-values for nuclear co-localisation of DAPI and A488 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and are analysed using Mann-Whitney test. **p<0.01, ****p≤0.0001 vs IL-1β. Scale bars: 100μm.*

After 24 hours of combined treatment with 10 ng/ml of IL-1β and with 1 or 10 μg/ml of IL-1Ra, the ciGEnCs displayed similar levels of *IL-6, IL-8, MCP-1, MIP-1α* and *GM-CSF* mRNA expression, compared to those of the untreated ciGEnCs (**Figure 4.7**). Changes in the mRNA expression of these genes were tested as in Chapter 3 their expression was shown to be affected in an IL-1β concentration-dependent manner (section 3.4.3, Figure 3.2).

The levels of mRNA expression of *IL-6*, *IL-8*, and *MCP-1* progressively decreased with increasing doses of IL-1Ra combined with 10 ng/ml of IL-1β (**Figure 4.7**). More specifically, a trend was observed for lower *IL-6* mRNA levels after treatment with 10 μg/ml of IL-1Ra (0.2 [0.1-0.3], P=0.09) compared to IL-1β treatments (0.7 [0.4-3.4]) from which the untreated ciGEnCs' *IL-6* levels (0.04 [0.004-0.24], P=0.002) were statistically significantly lower (**Figure 4.7-A**). *IL-8* mRNA expression was statistically significantly decreased following 10 μg/ml IL-1Ra treatment (0.43 [0.23-1.3], P=0.03) and that was also observed in untreated ciGEnCs (0.24 [0.018-1], P=0.004) compared to IL-1β treatment (4.5 [1.8-8.7]) (**Figure 4.7-B**).

For *MIP-1α*, a trend was observed for decreased mRNA expression levels after 10 μg/ml IL1-Ra treatments (0.0004 [0.0001-0.0007], P=0.09) compared to IL-1β treatments (0.002 [0.0004-0.004]). *MCP-1* mRNA levels after 10 μg/ml IL-1Ra treatments (0.05 [0.01-0.23], P=0.002) were decreased to reach expression levels similar to baseline expression levels of untreated ciGEnCs (0.05 [0.003-0.22], P=0.002) when compared to IL-1β-treated ciGEnCs (0.96 [0.34-2.14]) (**Figure 4.7-C**). For the *MIP-1α* and *GM-CSF* mRNA, only the 10 μg/ml of IL-1Ra led to expression levels similar to those of untreated ciGEnCs, although none of them were statistically significantly decreased (**Figure 4.7-D & 4.7-E**).

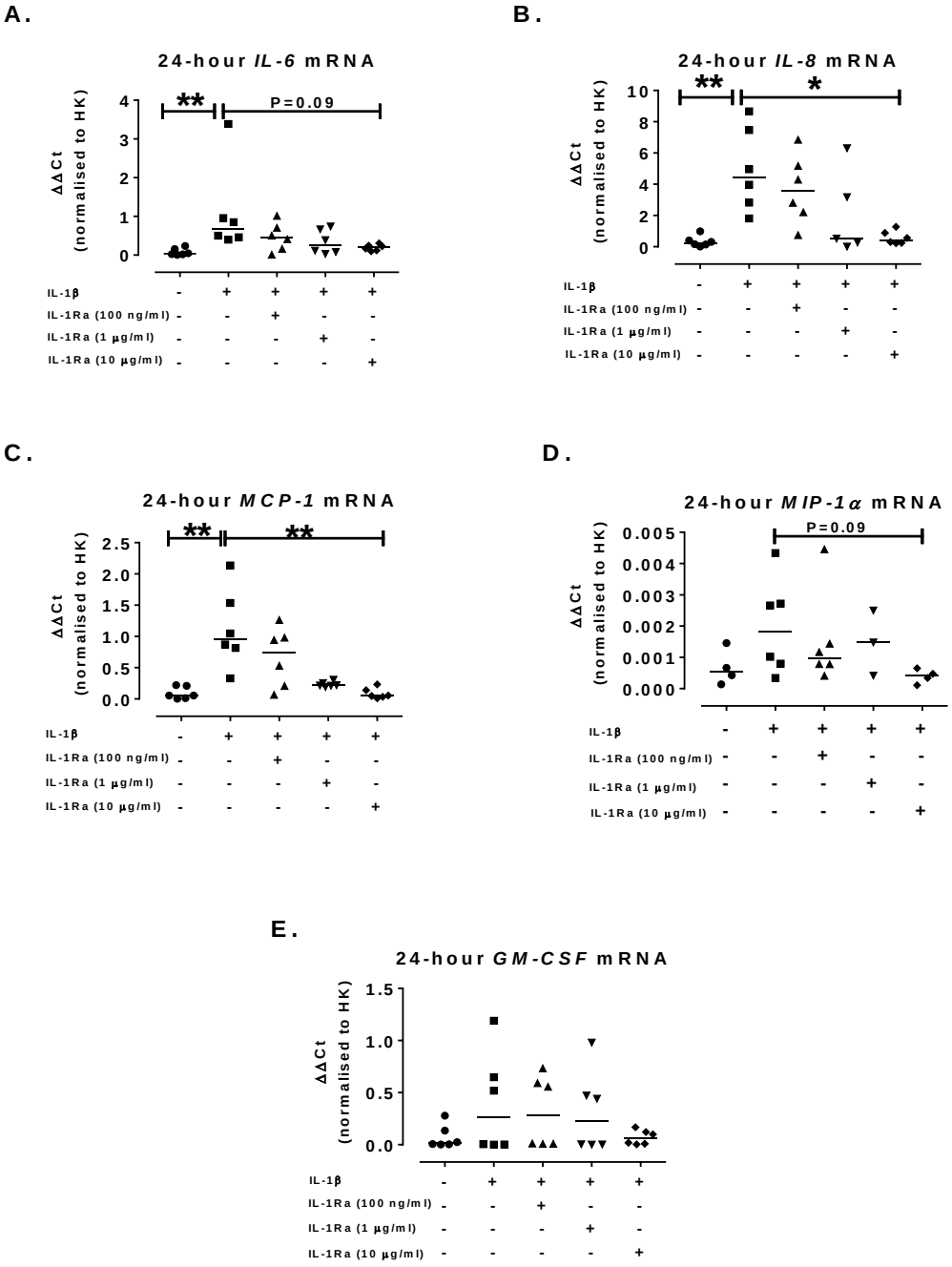

Figure 4.7 Effect of IL-1Ra treatments on IL-1β-mediated pro-inflammatory gene expression.

*Changes in the mRNA expression levels of IL-6 (A), IL-8 (B), MCP-1 (C), MIP-1α (D) and GM-CSF (E) following 24-hour treatments with IL-1β alone or with IL-1β together with 1 or 10 μg/ml of IL-1Ra. N=4/group. Data presented as median [range] are analysed using Kruskal-Wallis test, with Dunn's post-hoc test. *p≤0.05, **p≤0.01 vs untreated. HK: ACTB, TUBB, TBP.*

The effect of IL-1Ra (10 μg/ml) on IL-1β was also tested at the protein level and IL-6, IL-8, IL-10, GM-CSF and MIP-1α secreted protein levels of ciGEnCs treated with (IL-1β + 10 μg/ml IL-1Ra), were more similar to those of untreated ciGEnCs compared to those of IL-1β-treated ciGEnCs.

More specifically, the IL-1β-mediated secreted levels of IL-6 (5,445 pg/ml [1,639-5,815], p=0.003), IL-8 (5,697 pg/ml [4,871-5,866[, p=0.008), GM-CSF (1,518 pg/ml [234-1,626], p=0.003) and MIP-1α (398 pg/ml [382-406], p=0.005) were statistically significantly increased compared to untreated ciGEnCs (IL-6: 147 pg/ml [0-158]; IL-8: 1,714 pg/ml [325-1,887]); GM-CSF: 14 pg/ml [7-16] ; MIP-1α: 285 pg/ml [260-304]). The ciGEnCs treated with (IL-1β + 10 μg/ml IL-1Ra) exhibited IL-6 (620 pg/ml [276-765]), IL-8 (3,347 pg/ml [1,675-3,613]), GM-CSF (50 pg/ml [20-62]) and MIP-1α (326 pg/ml [304-328]) secretion levels that were closer although not as low as those of the untreated ciGEnCs.

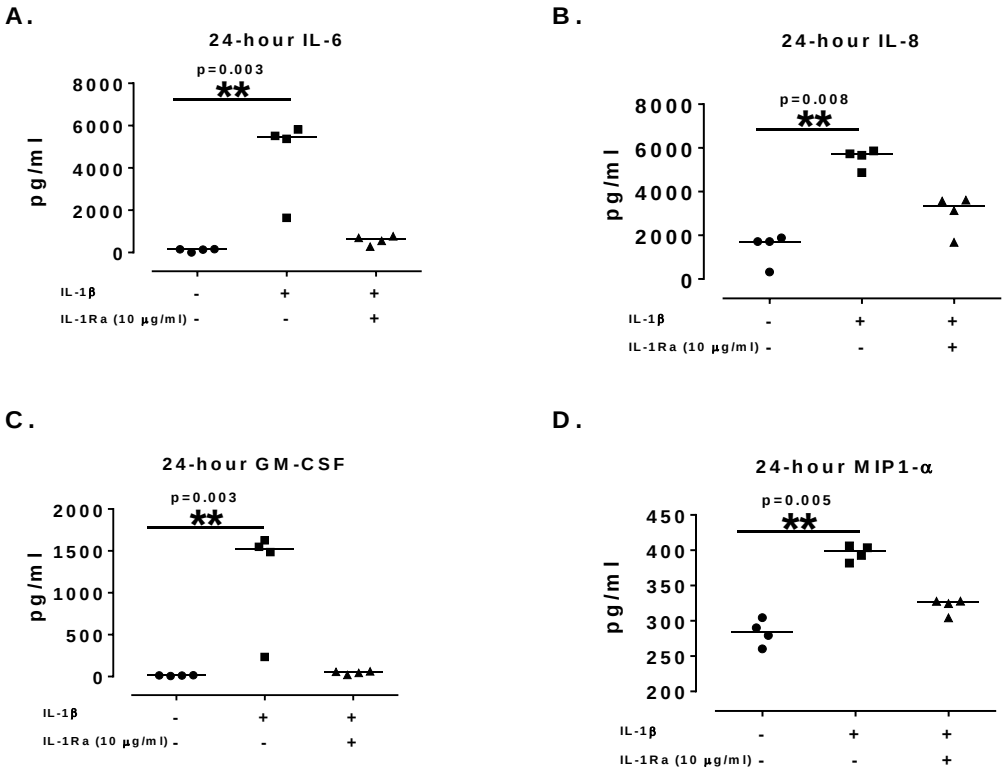

Figure 4.8 Effect of IL-1Ra treatments on IL-1β-mediated pro-inflammatory protein secretion.

*Changes in the secretion levels of IL-6 (A), IL-8 (B), IL-10 (C), IP-10 (D), MIP-1α (E) and M-CSF (F), GM-CSF (G) and TNF-α (H) following 24-hour treatments with IL-1β alone or with IL-1β together with 10 µg/ml of IL-1Ra. N=4/group. Data presented as (Mean +/- SEM) are analysed using Kruskal-Wallis test, with Dunn's post-hoc test. *p≤0.05, **p≤0.01 vs untreated.*

4.4. Results

4.4.1. Effect of LN patient sera on pro-inflammatory gene expression by ciGEnCs

The effect of 5% JSLE patient sera on ciGEnCs was assessed and compared to 5% healthy control sera after 4 and 24h. For the 24h serum incubations, the JSLE patients were stratified into active LN (renal BILAG A/B) and inactive LN (renal BILAG D/E) and compared to healthy controls.

4.4.1.1.ECM- and fibrosis-related genes

ECM deposition is an important pathological change seen in LN, the GFB is formed from contributions by both the GEnCs and the podocytes and remodeling of this will have a significant effect on the filtration properties of the glomerulus. To determine how JSLE patient sera leads to changes in the production of ECM components by GEnCs the expression of mRNA for genes involved in ECM remodeling were assessed.

No significant differences were seen in the mRNA for *COL1A1* after the comparison of HC serum treatments to JSLE serum treatments at 4h (**Figure 4.9-A**). At 24h, JSLE serum treatments significantly decreased *COL1A1* mRNA expression by the ciGEnCs (0.001 [0.0002-0.01], p=0.05) compared to HC serum treatments (0.003 [0.0006-0.007]) (**Figure 4.9-B)** but no statistically significant changes were observed when the JSLE serum treatments were distinguished onto active and inactive LN sera (**Figure 4.9-C**). No statistically significant changes occurred for *COL1A2* (**Figure 4.9-D-F**), *COL3A1* (**Figure 4.9-G-I**) or *COL4A1* (**Figure 4.9-J-L**) at 4 or 24h.

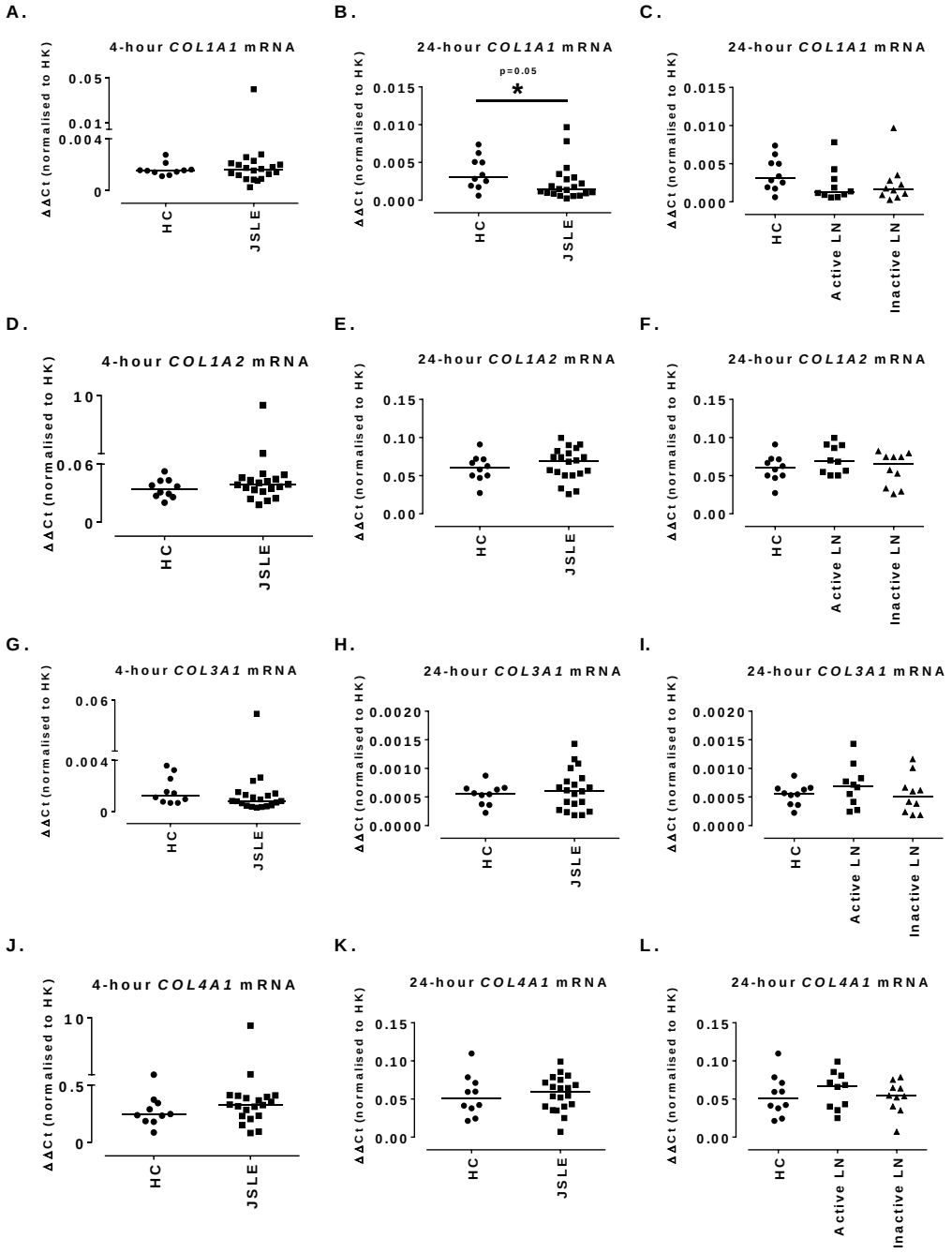

Figure 4.9 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on collagen mRNA expression.

*4-hour and 24-hour changes in COL1A1 (A, F), COL2A1 (B, G), COL3A1 (C, H), COL4A1 (D, I) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in COL1A1 (J), COL2A1 (K), COL3A1 (L), COL4A1 (M) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.*

Fibronectin (*FN*) mRNA was expressed at low levels following treatment with HC sera, no statistically significant differences occurred after 4h of JSLE serum incubation (**Figure 4.10-A**). At 24h, a trend was observed for increased *FN* mRNA expression after JSLE serum treatments (1.2 [0.7-1.6], p=0.06) compared to HC serum treatments (0.9 [0.8-1.4]) (**Figure 4.10-B**). When JSLE serum treatment were stratified into active and inactive LN disease serum treatments, no trend or statistical significance was observed (**Figure 4.10-C**).

Regarding the laminin genes, no statistically significant changes were observed for *laminin subunit beta 1* (*LAMB1*) mRNA expression following 4 or 24h stimulation (**Figure 4.10-D-F**). *Laminin subunit beta 2* (*LAMB2*) mRNA was expressed at low levels following treatment with HC sera, and no statistically significant differences occurred after 4h of JSLE serum incubation (**Figure 4.10-G**). *LAMB2* mRNA expression was statistically significantly increased by JSLE serum treatments (0.004 [0.002-0.007], p=0.04) compared to HCs (0.003 [0.0003-0.005]) (**Figure 4.10-H**). When distinguished into active and inactive LN disease serum treatments, the inactive serum treatments (0.004 [0.003-0.007], p=0.04) statistically significantly increased *LAMB2* expression compared to HCs (0.003 [0.0003-0.005]) while the active disease sera had no effect (**Figure 4.10-I**).

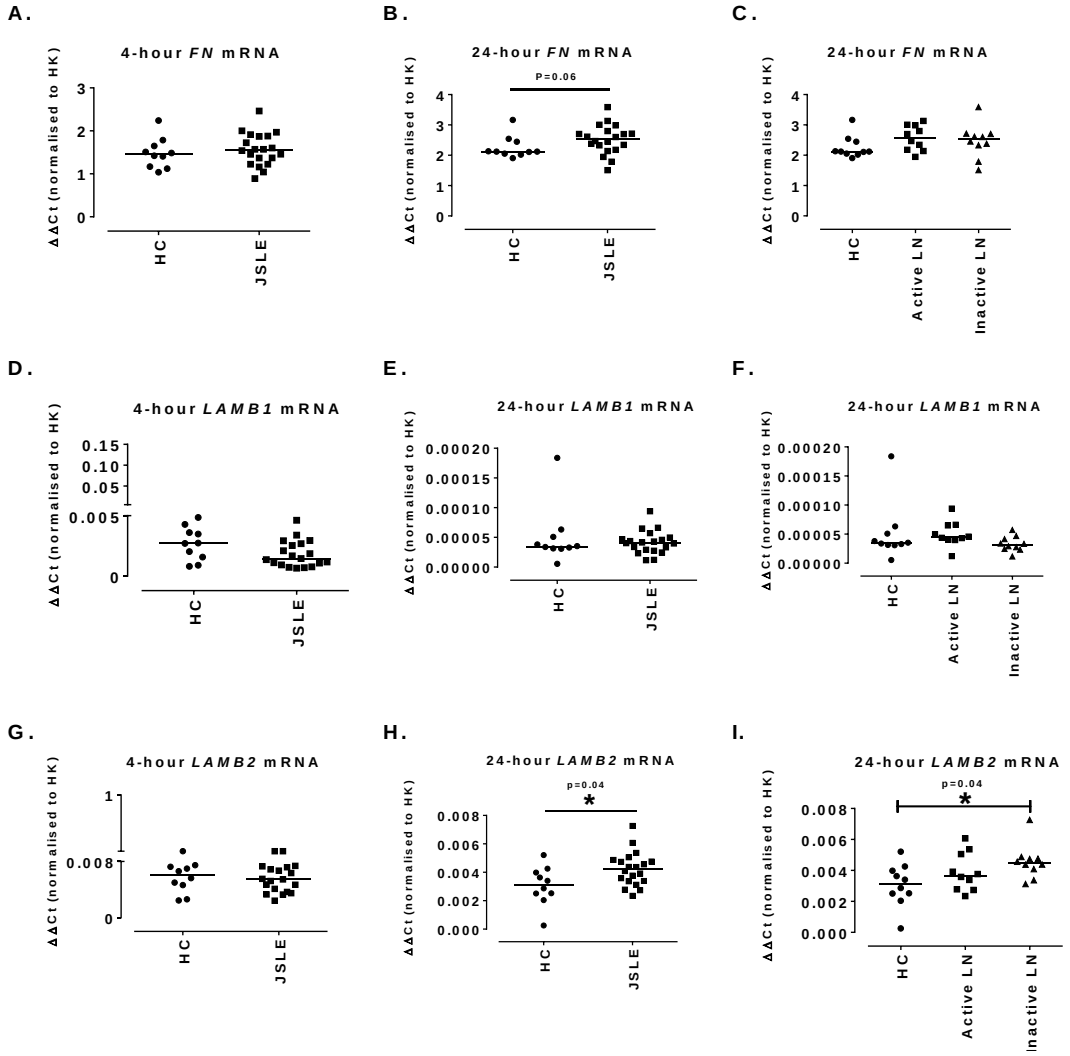

Figure 4.10 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on fibronectin and laminin mRNA expression.

*4-hour and 24-hour changes in FN (A, D), LAMB1 (B, E), LAMB2 (C, F) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in FN (G), LAMB1 (H), LAMB2 (I) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.*

No statistically significant changes were seen in the mRNA expression of *tissue inhibitor of metalloproteinases 1* (*TIMP1*) (**Figure 4.11-A-C**) or *matrix metalloproteinase (MMP) 2* (*MMP2*) (**Figure 4.11-D-F**) following ciGEnCs' treatments with JSLE sera compared to HCs, at either 4h or 24h. A trend was observed (p=0.06) for decreased ciGEnC expression of *MMP7* after the 4h JSLE serum treatments (0.4 [0.1-1.7]) compared to HC treatments (0.9 [0.3-1.8]) (**Figure 4.11-G**) but no remarkable changes were observed at 24h (**Figure 4.11-H & I**). No statistically significant differences were observed for *MMP7* by JSLE sera compared to HC sera at either 4h or 24h (**Figure 4.11-A**) or MMP9 at any time point (**Figure 4.11-J-L**).

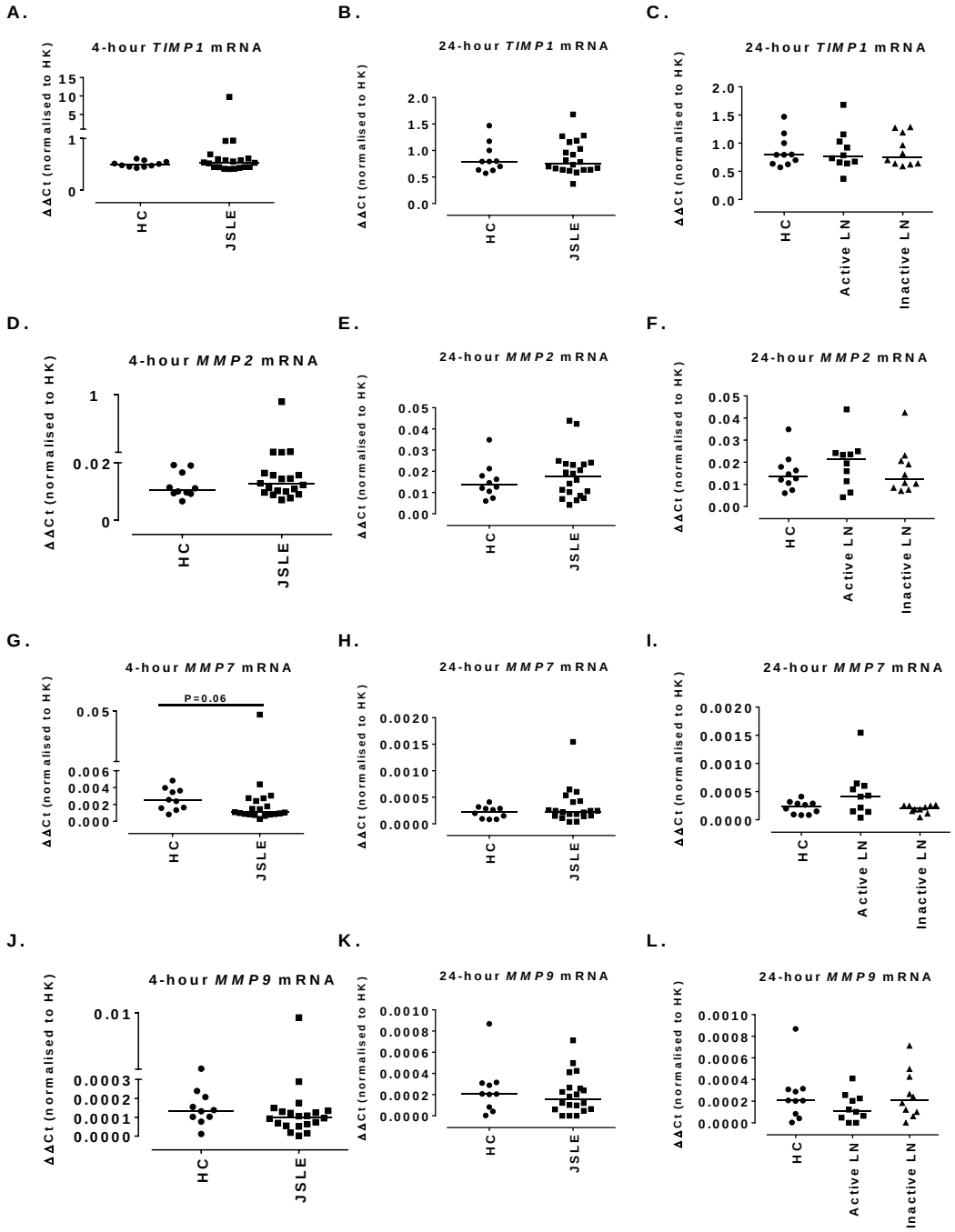

Figure 4.11. Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on TIMP1 and MMPs mRNA expression.

4-hour and 24-hour changes in TIMP1 (A, F), MMP2 (B, G), MMP7 (C, H), MMP9 (D, I) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in TIMP1 (J), MMP2 (K) MMP7 (L), MMP9 (M) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. p=NS, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.

4.4.1.2. Pro-inflammatory mediators

The expression of pro-inflammatory genes and subsequent production of pro-inflammatory proteins by the human GEnCs was tested following cytokine and LPS treatments, and data presented in Chapter 3. As statistically significant changes were observed following certain stimuli, the effect of serum treatments on the same genes was tested and is presented in this Chapter.

At 4h, no statistically significant differences were observed for *TNF-α* mRNA levels (**Figure 4.12-A**). The 24h JSLE serum treatments did not modify *TNF-α* mRNA levels compared to HCs (**Figure 4.12-B**), however when distinguished into active and inactive LN serum treatments the latter significantly decreased *TNF-α* mRNA expression (0.0003 [0.0001-0.0009], p=0.03) compared to HC serum treatments (0.0008 [0.0004-0.002]) (**Figure 4.12-C**).

No statistically significant differences were observed at 4h for *IL-1β* mRNA expression when the HC effect was compared to the JSLE serum effect (**Figure 4.12-D**). A trend was observed for increased *IL-1β* mRNA expression after JSLE serum treatments (0.003 [0.0008-0.006], p=0.07]) compared to HCs (0.002 [0.0008-0.004]) (**Figure 4.12-E**), however no statistically significant change occurred when the JSLE serum treatments were distinguished into active and inactive LN (**Figure 4.12-F**).

No changes in *IL-6* levels were observed at 4h of HC and JSLE serum treatments (**Figure 4.12-G**). JSLE serum treatments (0.002 [0.0007-0.003], p=0.03) significantly increased *IL-6* mRNA expression compared to HCs (0.001 [0.0006-0.002]) (**Figure 4.12-H**) and a trend was observed for increased *IL-6* mRNA expression following active LN serum treatments (0.002 [0.0006-0.002], p=0.06) compared to HCs (0.001 [0.0006-

0.002]) (**Figure 4.12-KI**). Finally, no statistically significant changes were observed for *IL-10* expression at either 4 or 24h (**Figure 4.12-J-L**).

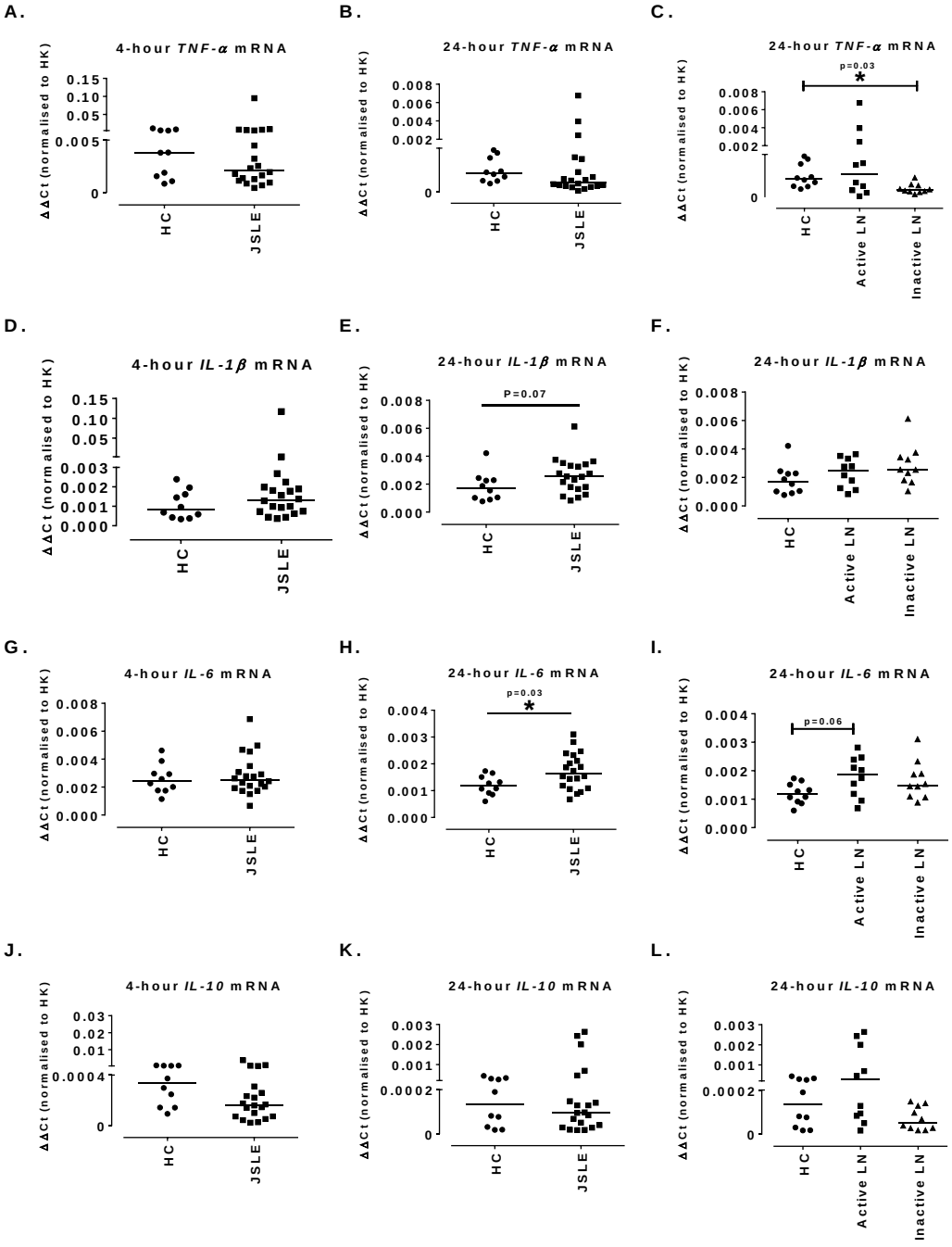

Figure 4.12 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on cytokine mRNA expression.

*4-hour and 24-hour changes in TNF-α (A, F), IL-1β (B, G), IL-6 (C, H), IL-10 (D, I) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in TNF-α (J), IL-1β (K) IL-6 (L), IL-8 (M) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.*

Among the chemokine genes tested, no statistically significant differences were observed for *IL-8* (**Figure 4.13-A-C**) and *MIP-1α* (**Figure 4.13-D-F**) mRNA expression following HC and JSLE serum treatments at 4 and 24h and no differences were observed at 24h when the JSLE serum treatments were distinguished into active and inactive LN.

At 4h, no statistically significant changes in *IP-10* mRNA levels were observed between HC and JSLE serum treatments (**Figure 4.13-G**). A trend was observed for decreased *IP-10* mRNA expression following JSLE serum (0.00005 [0.000004-0.0005], p=0.055) treatments compared to HCs (0.0001 [0.00003-0.0008]) (**Figure 4.13-H**). When JSLE serum was distinguished into active and inactive LN serum, the inactive LN serum treatments (0.00005 [0.00002-0.0001], p=0.04) significantly decreased *IP-10* mRNA levels compared to HCs (0.0001 [0.00003-0.0008]) but no significant difference was demonstrated between HCs and active LN serum treatments (0.0001 [0.000004-0.0005]) (**Figure 4.13-I**). *IP-10* mRNA, however, was in general very lowly expressed and therefore no absolute conclusions should be drawn from these findings.

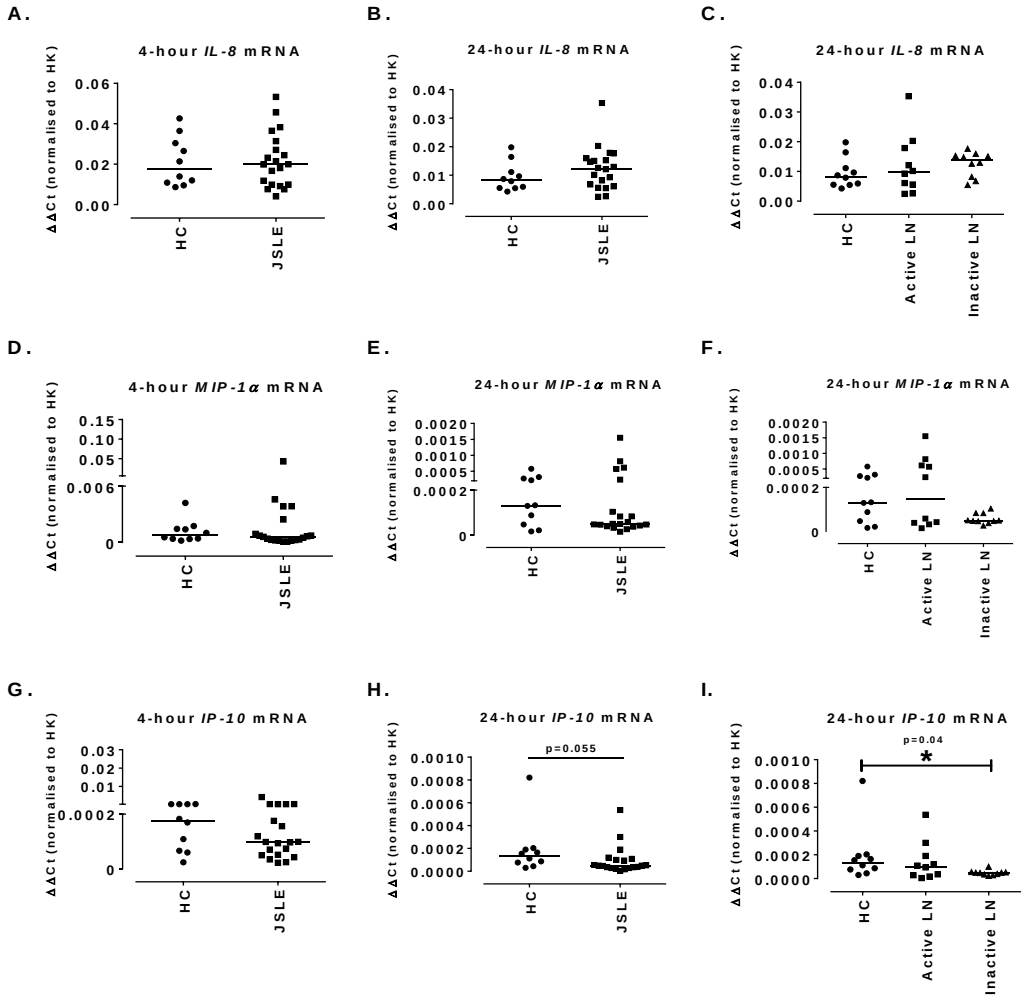

Figure 4.13 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on chemokine mRNA expression.

*4-hour and 24-hour changes in IL-8 (A, D), MIP-1α (B, E), IP-10 (C, F) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in IL-8 (G), MIP-1α (H) IP-10 (I) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.*

No statistically significant differences were observed in the mRNA expression levels of urinary biomarkers for childhood LN activity (*MCP-1*, *VCAM-1*, *L-PGDS*, *TF*) at either 4h or 24h following HC and JSLE serum treatments or HC and active/inactive LN serum treatments, with *TF* and *L-PGDS* demonstrating negligible expression levels **Figure 4.14**).

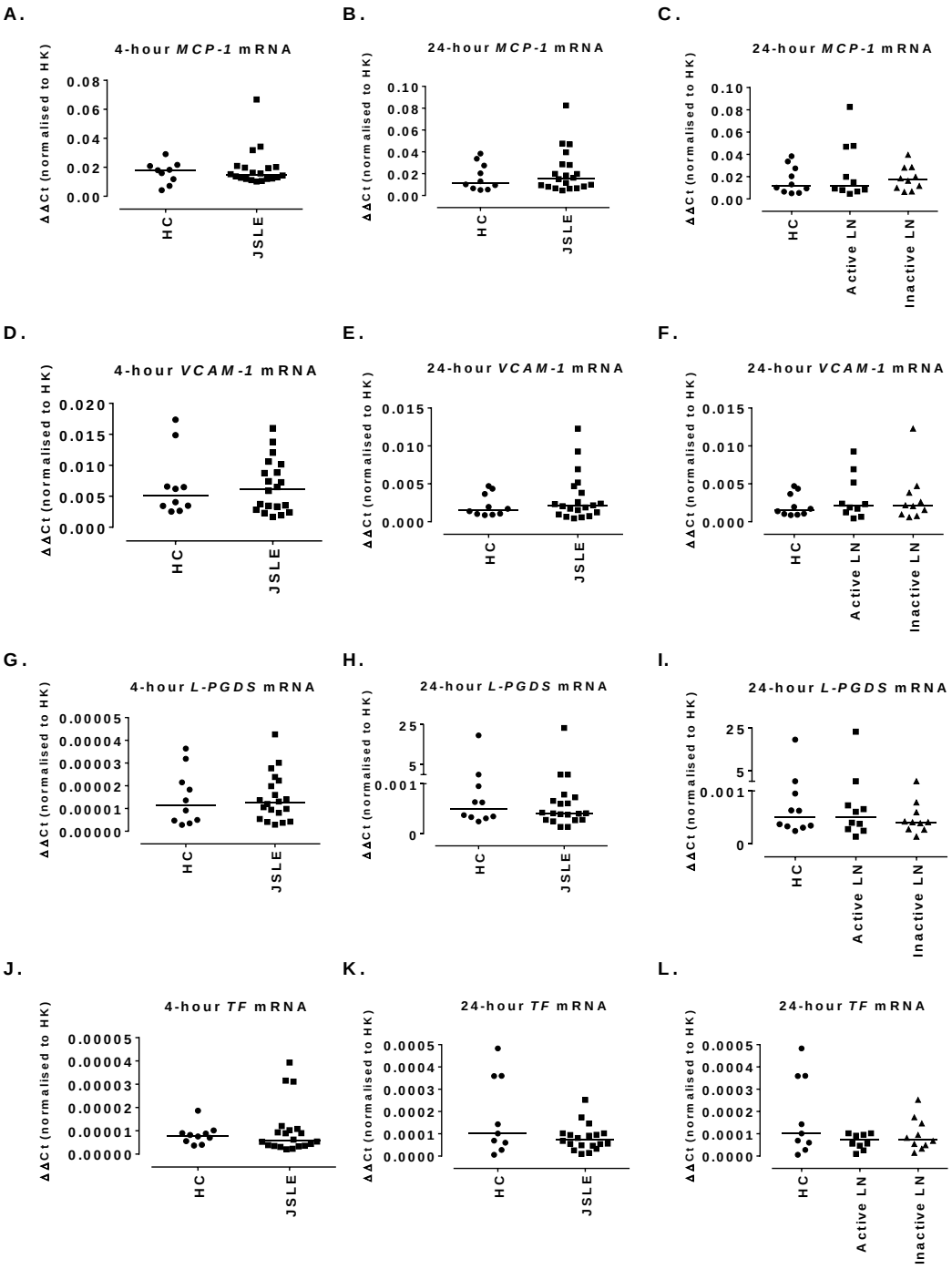

Figure 4.14 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on urinary biomarker mRNA expression.

4-hour and 24-hour changes in MCP-1 (A, F), VCAM-1 (B, G), TF (C, H), L-PGDS (D, I) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in MCP-1 (J), VCAM-1 (K) TF (L), L-PGDS (M) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. p=NS, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.

M-CSF (**Figure 4.15-A-C**) and *GM-CSF* (**Figure 4.15-D-F**) mRNA expression by ciGEnCs following 4h incubation with sera taken from HC was not affected by treatment with sera from JSLE patients. Levels of *M-CSF* and *GM-CSF* mRNA were similar at 24h and again were unaffected by sera treatments. Further stratification of JSLE patients into active and inactive LN revealed no differences between groups (**Figure 4.15**).

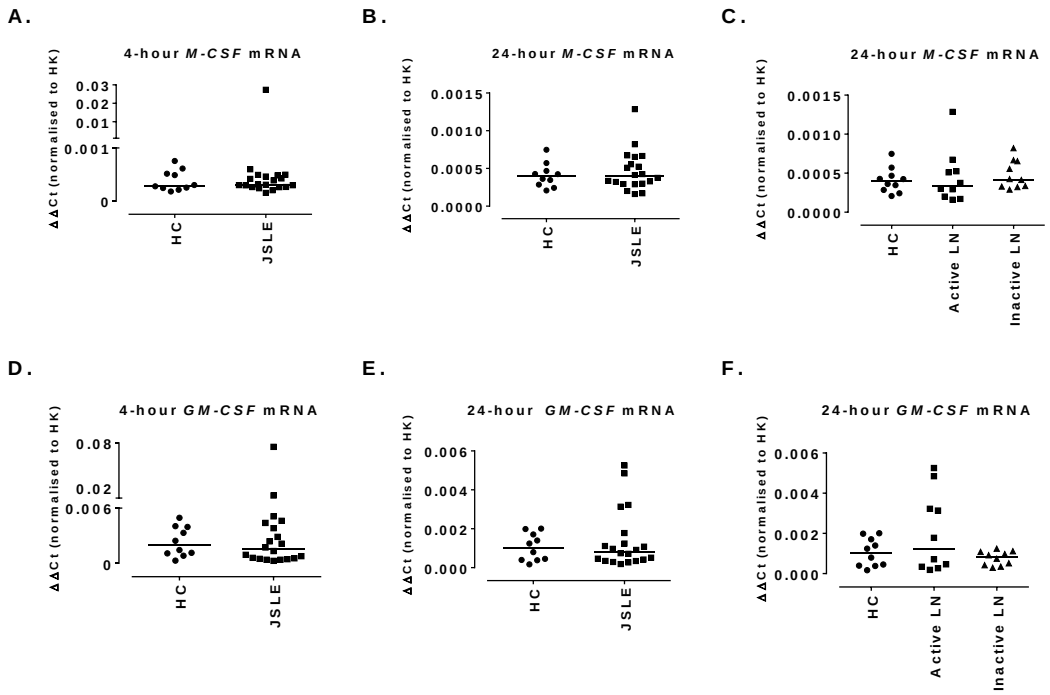

Figure 4.15 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on blood cell growth factor mRNA expression.

4-hour and 24-hour changes in M-CSF (A, C), GM-CSF (B, D) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in M-CSF (E), GM-CSF (F) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. p=NS, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.

Similarly, *TNFR1* (**Figure 4.16-A-C**), *TNFR2* (**Figure 4.16-D-F**), *IL-1RI* (**Figure 4.16-G-I**) and *IL-1RII* (**Figure 4.16-J-L**) mRNA expression by ciGEnCs following 4h incubation with HC sera was not affected by JSLE serum treatments. Levels of *TNFR1*, *TNFR2*, *IL-1RI* and *IL-1RII* were similar at 24h and again were unaffected by different serum treatments. Further stratification of JSLE patients into active and inactive LN revealed no differences between groups (**Figure 4.16**).

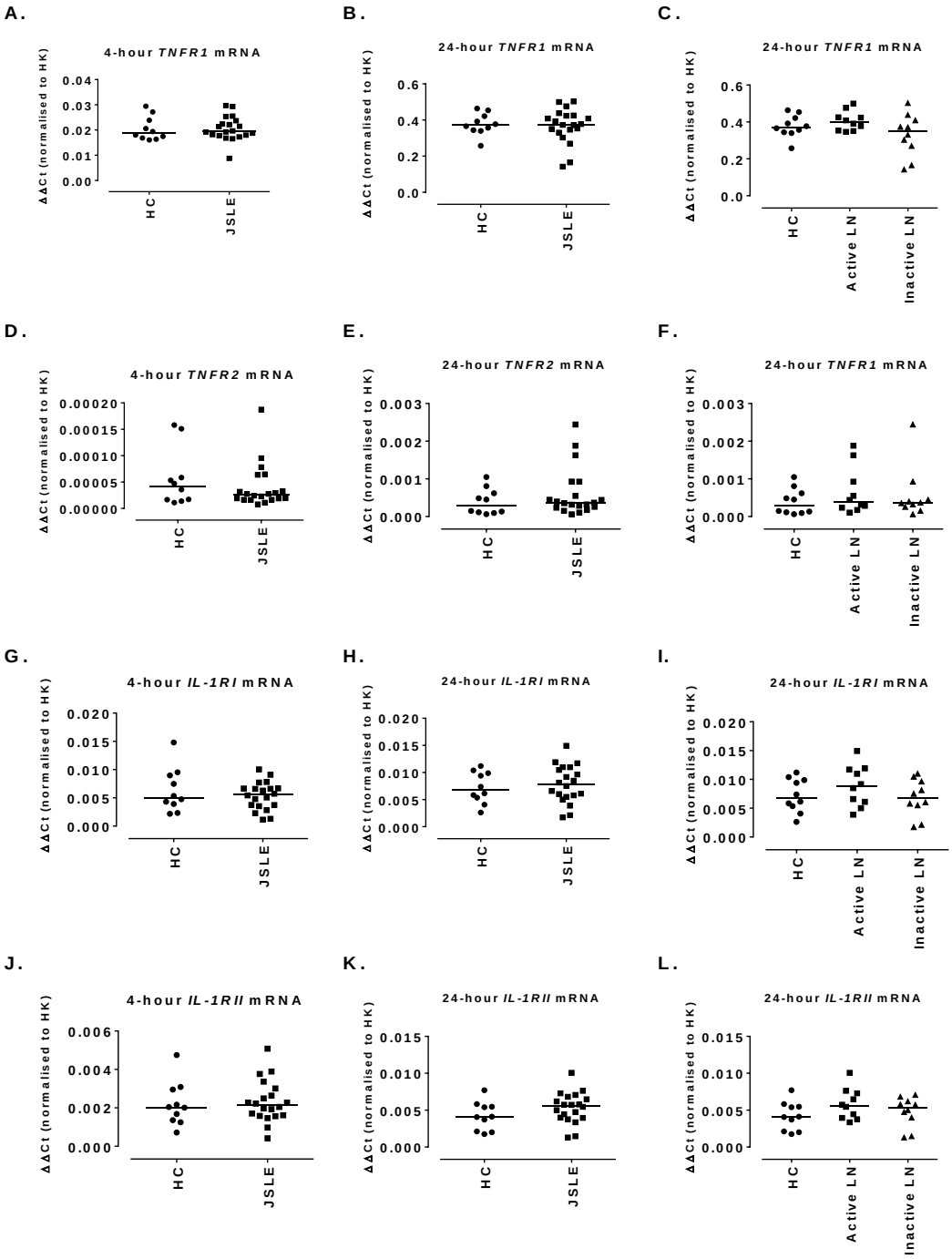

Figure 4.16 Effect of 4-hour and 24-hour ciGEnC treatments with 5% concentrations of JSLE or HC sera on TNF-α and IL-1β receptor mRNA expression.

4-hour and 24-hour changes in TNFR1 (A, F), TNFR2 (B, G), IL-1RI (C, H), IL-1RII (D, I) mRNA expression levels; comparisons between HC and JSLE serum treatments. 24-hour changes in TNFR1 (J), TNFR2 (K) IL-1RI (L), IL-1RII (M) mRNA expression levels; comparisons of HC serum treatments with active and inactive LN serum treatments. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test or Kruskal-Wallis with Dunn's post-hoc test. p=NS, JSLE serum vs HC/active or inactive LN serum vs HC. N=10-20/group. HK: ACTB, TUBB.

In general, the 4h serum treatments did not induce any significant changes in the mRNA expression levels between JSLE- and HC-treated groups whereas the 24h serum treatments led to more trends or significant changes in the mRNA expression of the genes tested.

4.4.2. Effect of human neutrophil conditioned media on pro-inflammatory gene production by GEnCs

Neutrophils isolated from healthy adult blood donors were stimulated for 1h with TNF-α, IFN-γ and LPS and their conditioned media together with those of untreated neutrophils were collected and used to incubate ciGEnCs for 24h in order to test potential changes in pro-inflammatory gene expression. Untreated, TNF-α, IFN-γ- and LPS-treated ciGEnCs were included at all times and were compared to their neutrophil conditioned media counterparts in order to evaluate the potential effect of the neutrophil conditioned media on ciGEnCs and to ensure that this effect was not just a consequence of cytokine and LPS treatments.

4.4.2.1. Cytokine and Chemokine mRNA expression after treatment with neutrophil conditioned media

ciGEnCs expressed low levels of mRNA for *IL-6* (**Figure 4.17-A**), *IL-8* (**Figure 4.17-B**), *MIP-1α* (**Figure 4.17-C**), and *IP-10* (**Figure 4.17-D**), under basal conditions (untreated ciGEnCs), and no significant change was noted following incubation with conditioned media from untreated neutrophils. When treated with conditioned media from TNF-α-, IFN-γ- and LPS-activated neutrophils the ciGEnC expression levels of *IL-6*, *IL-8*, *MIP-1α* and *IP-10* did not differ from those of the TNF-α-, IFN-γ- and LPS-treated ciGEnCs.

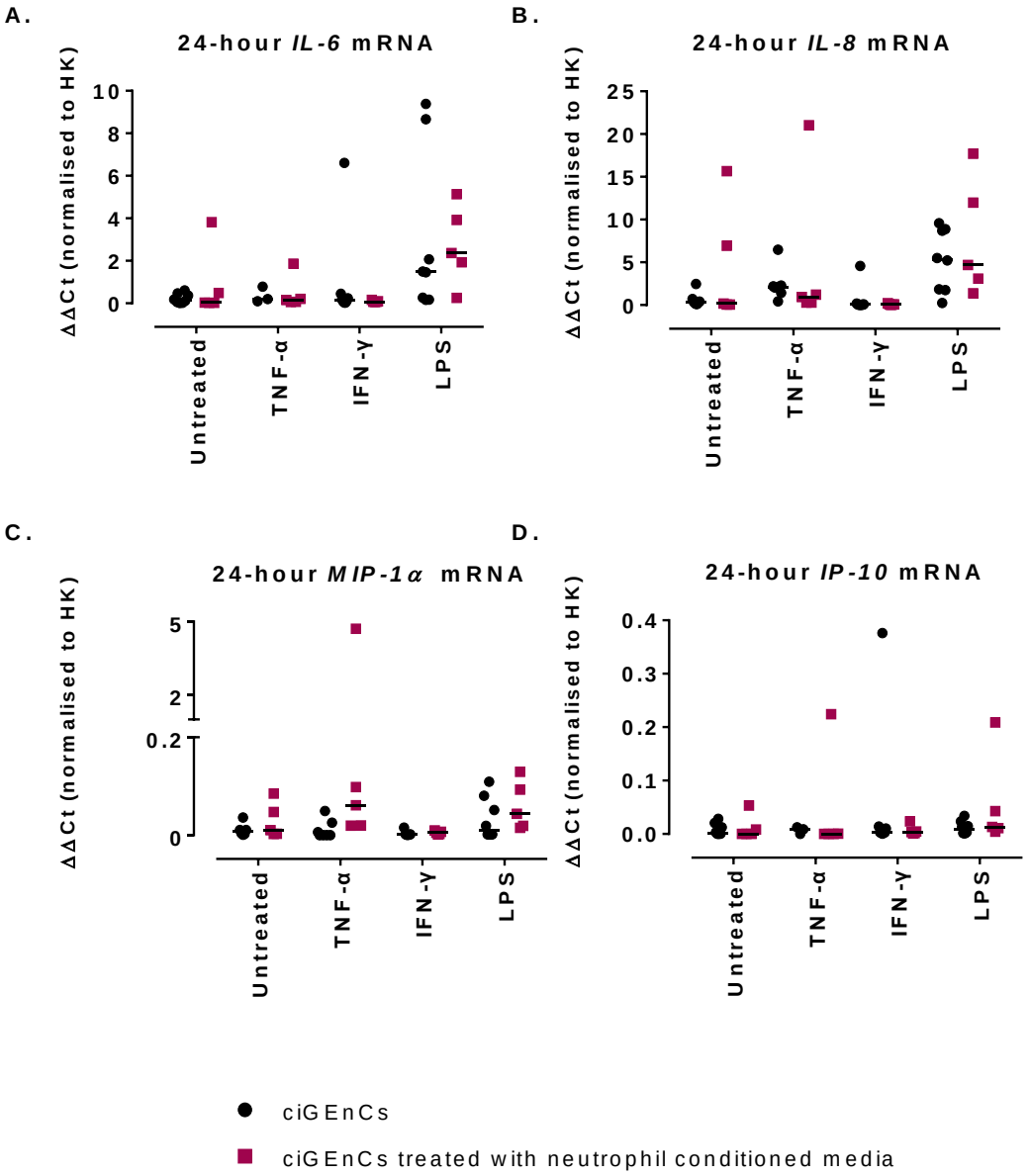

Figure 4.17 Effect of 24-hour ciGEnC treatments with untreated, TNF-α-, IFN-γ- and LPS-treated human neutrophil conditioned media on cytokine and chemokine mRNA expression.

Changes in IL-6 (A), IL-8 (B), MIP-1α (C), IP-10 (D) mRNA expression levels. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs neutrophil conditioned media-treated ciGEnCs: Untreated, TNF-α, IFN-γ, LPS). P=NS. N=3-8/group. HK: ACTB, TUBB, TBP.

4.4.2.2.Urinary biomarker mRNA expression after treatment with neutrophil conditioned media

No statistically significant changes were observed for *MCP-1* (**Figure 4.18-A**) and *VCAM-1* (**Figure 4.18-B**) mRNA expression when untreated ciGEnCs were compared to ciGEnCs stimulated with untreated neutrophil conditioned media. When treated with conditioned media from TNF-α-, IFN-γ- and LPS-activated neutrophils the ciGEnC expression levels of *MCP-1* and *VCAM-1* did not differ from those of the TNF-α-, IFN-γ- and LPS-treated ciGEnCs (**Figure 4.18**).

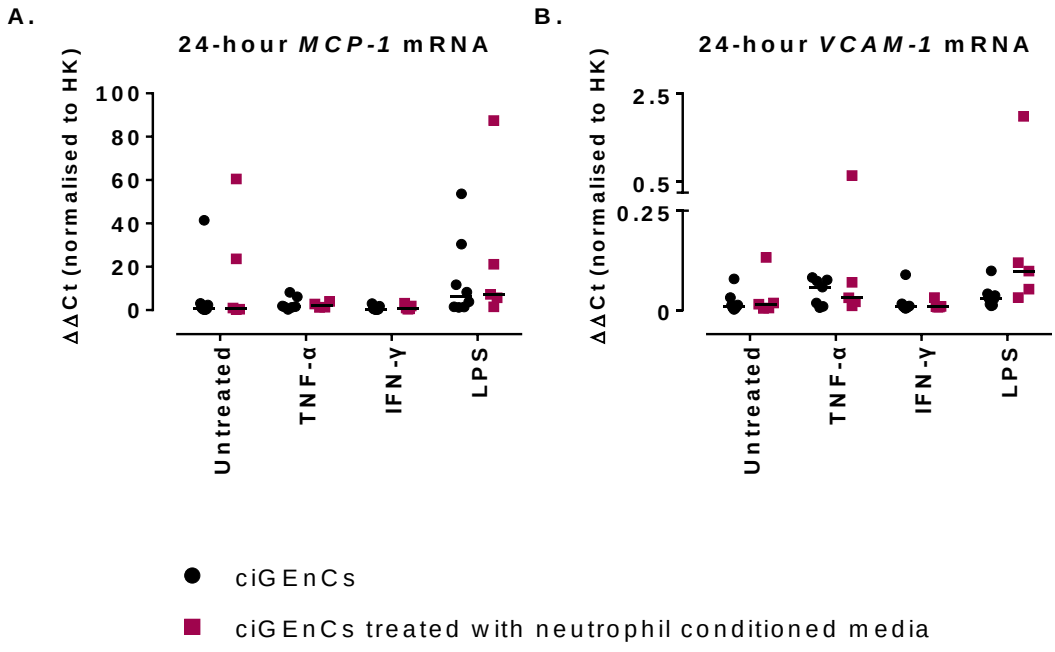

Figure 4.18 Effect of 24-hour ciGEnC treatments with untreated, TNF-α-, IFN-γ- and LPS-treated human neutrophil conditioned media on urinary biomarker mRNA expression.

Changes in MCP-1 (A), VCAM-1 (B) mRNA expression levels. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs neutrophil conditioned media-treated ciGEnCs: Untreated, TNF-α, IFN-γ, LPS). P=NS. N=3-8/group. HK: ACTB, TUBB, TBP.

4.4.2.3.Blood cell growth factors mRNA expression after treatment with neutrophil conditioned media

ciGEnCs expressed low levels of mRNA for *M-CSF* (**Figure 4.19-A**) and *GM-CSF* (**Figure 4.19-B**), under basal conditions (untreated ciGEnCs), and no significant change was noted following incubation with conditioned media from untreated neutrophils. When treated with conditioned media from TNF-α-, IFN-γ- and LPS-activated neutrophils the ciGEnC expression levels of *M-CSF* and *GM-CSF* did not differ from those of the TNF-α-, IFN-γ- and LPS-treated ciGEnCs (**Figure 4.19**).

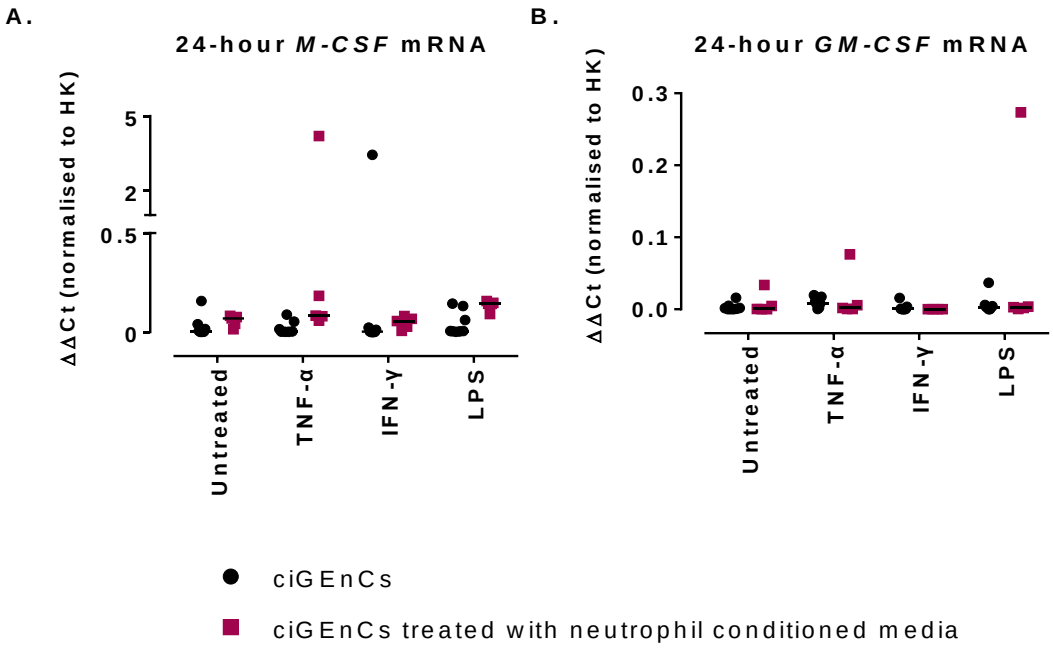

Figure 4.19 Effect of 24-hour ciGEnC treatments with untreated, TNF-α-, IFN-γ- and LPS-treated human neutrophil conditioned media on blood cell growth factor mRNA expression.

Changes in M-CSF (A), GM-CSF (B) mRNA expression levels. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs neutrophil conditioned media-treated ciGEnCs: Untreated, TNF-α, IFN-γ, LPS). P=NS. N=3-8/group. HK: ACTB, TUBB, TBP.

4.4.2.4.Cytokine receptors mRNA expression after treatment with neutrophil conditioned media

No statistically significant changes were observed for *TNFR1* (**Figure 4.20-A**), *TNFR2* (**Figure 4.20-B**), *IL-1RI* (**Figure 4.20-C**) and *IL-1RII* (**Figure 4.20-D**) mRNA expression when untreated ciGEnCs were compared to ciGEnCs stimulated with untreated neutrophil conditioned media. When treated with conditioned media from TNF-α-, IFN-γ- and LPS-activated neutrophils the ciGEnC expression levels of *TNFR1*, *TNFR2*, *IL-1RI* and *IL-1RII* did not differ from those of the TNF-α-, IFN-γ- and LPS-treated ciGEnCs (**Figure 4.20**).

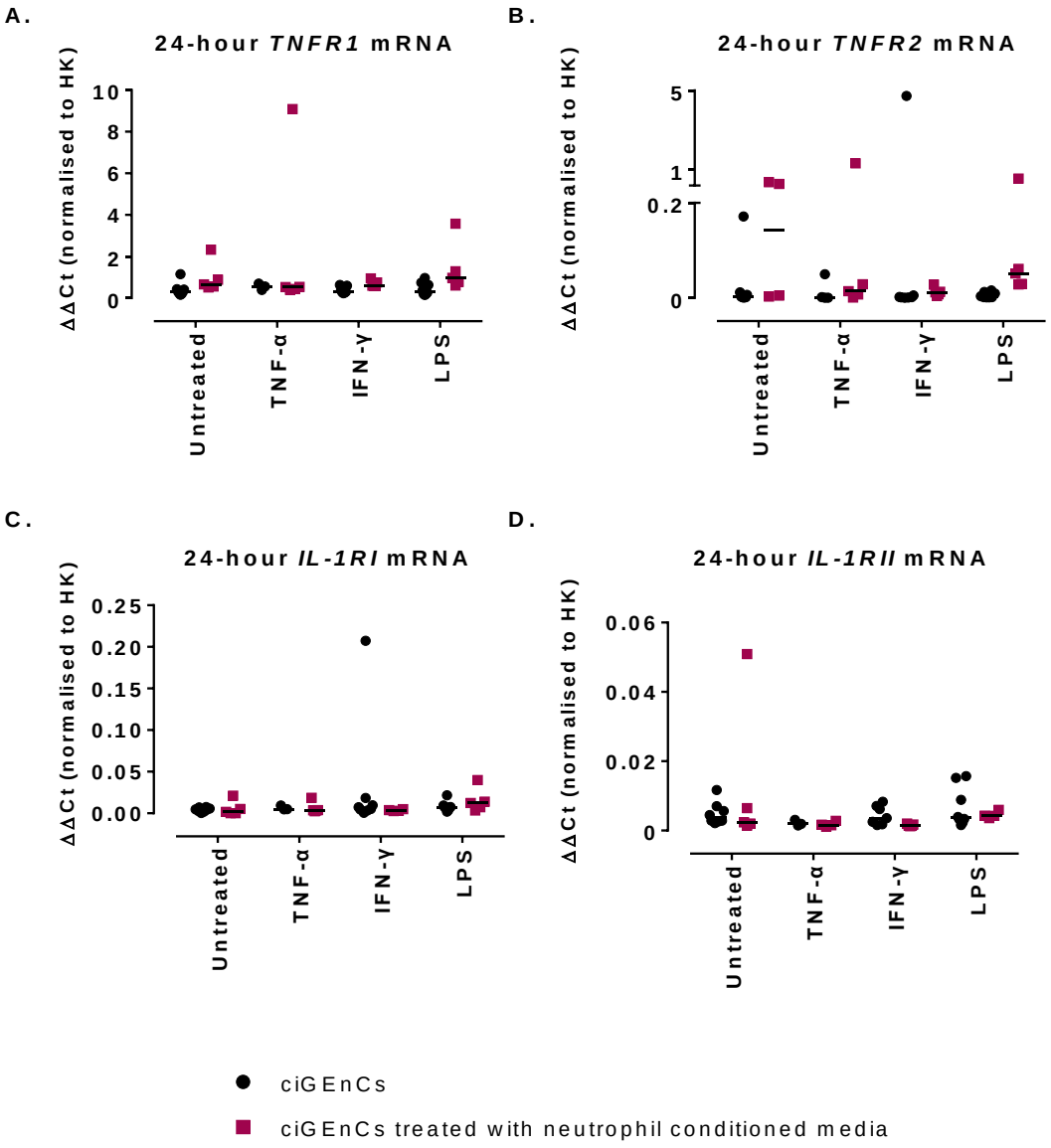

Figure 4.20 Effect of 24-hour ciGEnC treatments with untreated, TNF-α-, IFN-γ- and LPS-treated human neutrophil conditioned media on TNF-α and IL-1β receptor mRNA expression.

*Changes in TNFR1 (A), TNFR2 (B), IL-1RI (C), IL-1RII (D) mRNA expression levels. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (Untreated, TNF-α, IFN-γ, LPS). *p≤0.013, ***p≤0.00075. N=3-8/group. HK: ACTB, TUBB, TBP.*

4.4.3. Effect of human macrophage conditioned media on the pro-inflammatory gene production by GEnCs

The effect of human macrophage conditioned media on ciGEnCs was explored. However, due to low cell number generated by primary human macrophages, an *in vitro* THP-1 macrophage/ciGEnC model was also developed.

Human monocytes were isolated from healthy adult blood donors and differentiated into macrophages (MΦ) using M-CSF. Initially, the MΦ were treated for 48h with IFN-γ and LPS. Subsequently the untreated, IFN-γ and LPS MΦ conditioned media were collected and used to treat ciGEnCs for 24h in order to explore potential changes in *MCP-1*, *VCAM-1*, *IL-6* and *IL-8* mRNA expression (these pro-inflammatory genes were used to initially perform a pilot assay in order to determine whether a substantial effect would be exerted on the ciGEnCs by the MΦ conditioned media) (**Figure 4.21**). Among all MΦ media treatments, the LPS MΦ media had the most prominent effect on ciGEnCs. The LPS MΦ media significantly increased *MCP-1* mRNA expression (3 [2-4], p=0.01) compared to untreated ciGEnCs (0.1 [0.07-0.2]) (**Figure 4.21-A**) and similarly the LPS MΦ media significantly increased *VCAM-1* mRNA levels (0.1 [0.02-0.2], p=0.01) compared to untreated ciGEnCs (0.01 [0.01-0.02]) (**Figure 4.21-B**) and *IL-6* mRNA levels (0.9 [0.6-1], p=0.0006) compared to untreated ciGEnCs (0.02 [0.008-0.03]) (**Figure 4.21-C**). Although not statistically significantly, the LPS MΦ media considerably elevated *IL-8* mRNA levels (1 [0.4-2]) compared to untreated ciGEnCs (0.03 [0.02-0.05]) (**Figure 4.21-D**).

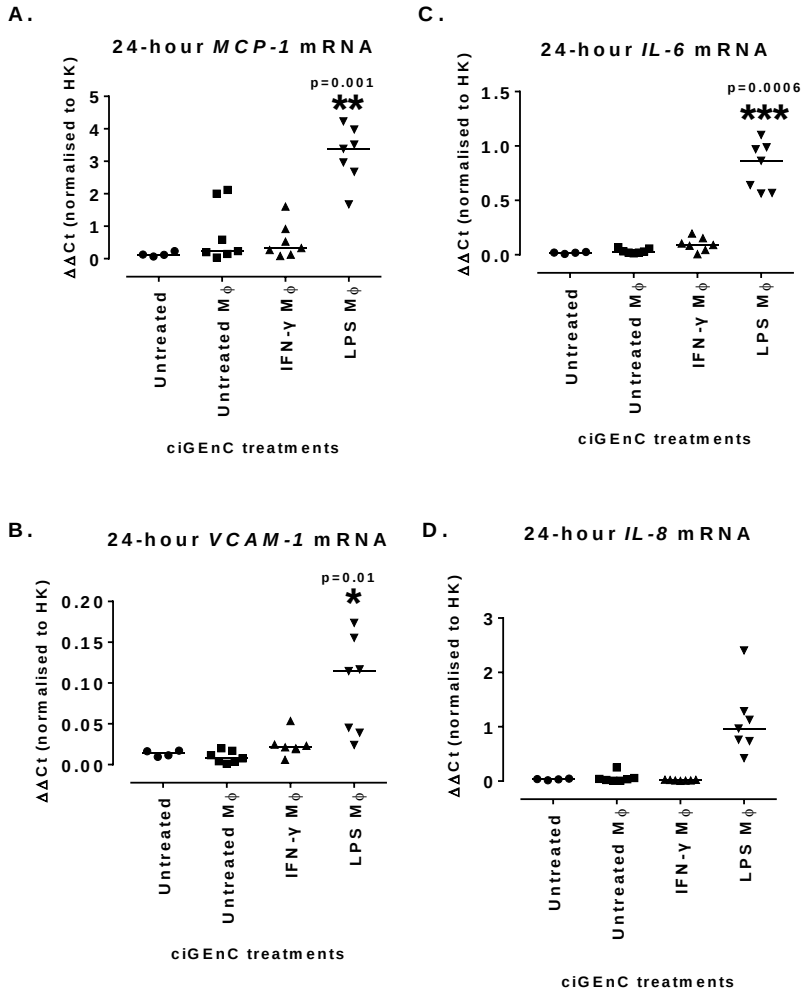

Figure 4.21 Exploring the effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated human macrophage conditioned media on MCP-1, VCAM-1, IL-6 and IL-8 gene expression.

*Changes in MCP-1 (A), VCAM-1 (B), IL-6 (C) and IL-8 mRNA expression. Data presented as median ΔΔCt [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05, **p≤0.01, ***p≤0.001, vs Untreated. N=4-6/group. HK: TBP, TUBB.*

Treatment of ciGEnCs with conditioned media from MΦ treated with LPS induced an increase in the production of pro-inflammatory genes including *MCP-1*, *VCAM-1, IL-6* and *IL-8*. It was therefore hypothesised that as well as a direct LPS effect on ciGEnCs, LPS could induce the secretion of an inflammatory mediator that activates GEnCs, such as the prototypical pro-inflammatory cytokine TNF-α.

To test this hypothesis healthy adult donor blood samples were collected, and isolated monocytes were differentiated into human macrophages. These were treated with LPS for 24h with or without a 2h addition of anti-TNF-αAb (Ab) at 22h at 37ºC and 5% CO_2, before the conditioned media were collected and used to stimulate ciGEnCs for 24 h. Following stimulation, the ciGEnCs were lysed in Trizol for RNA extraction.

4.4.3.1.Cytokine and chemokine mRNA expression after treatment with MΦ conditioned media

The LPS MΦ media significantly increased *IL-6* mRNA expression (1.3 [0.4-2], p=0.04) compared to untreated ciGEnCs (0.02 [0.01-0.05]) (**Figure 4.22-A**). However, anti-TNF-α Ab addition did not abolish the LPS MΦ effect (1.2 [0.4-1]) (**Figure 4.22-A**), possibly because LPS itself has a strong effect on *IL-6* mRNA expression. For *IL-8* mRNA expression, no statistically significant values occurred (**Figure 4.22-B**). *MIP-1α* mRNA levels were not substantially affected by LPS MΦ media stimulation and therefore, no remarkable changes occurred (**Figure 4.22 -C**). A trend was observed for increased *IP-10* mRNA expression after LPS MΦ media treatment (0.005 [0.0005-0.006], p=0.06) compared to untreated ciGEnCs (0.0002 [0.0002-0.0004 (**Figure 4.22-D**). This could be attributed to the well-established effect of LPS on *IP-10* expression (**Figure 4.22-D**).

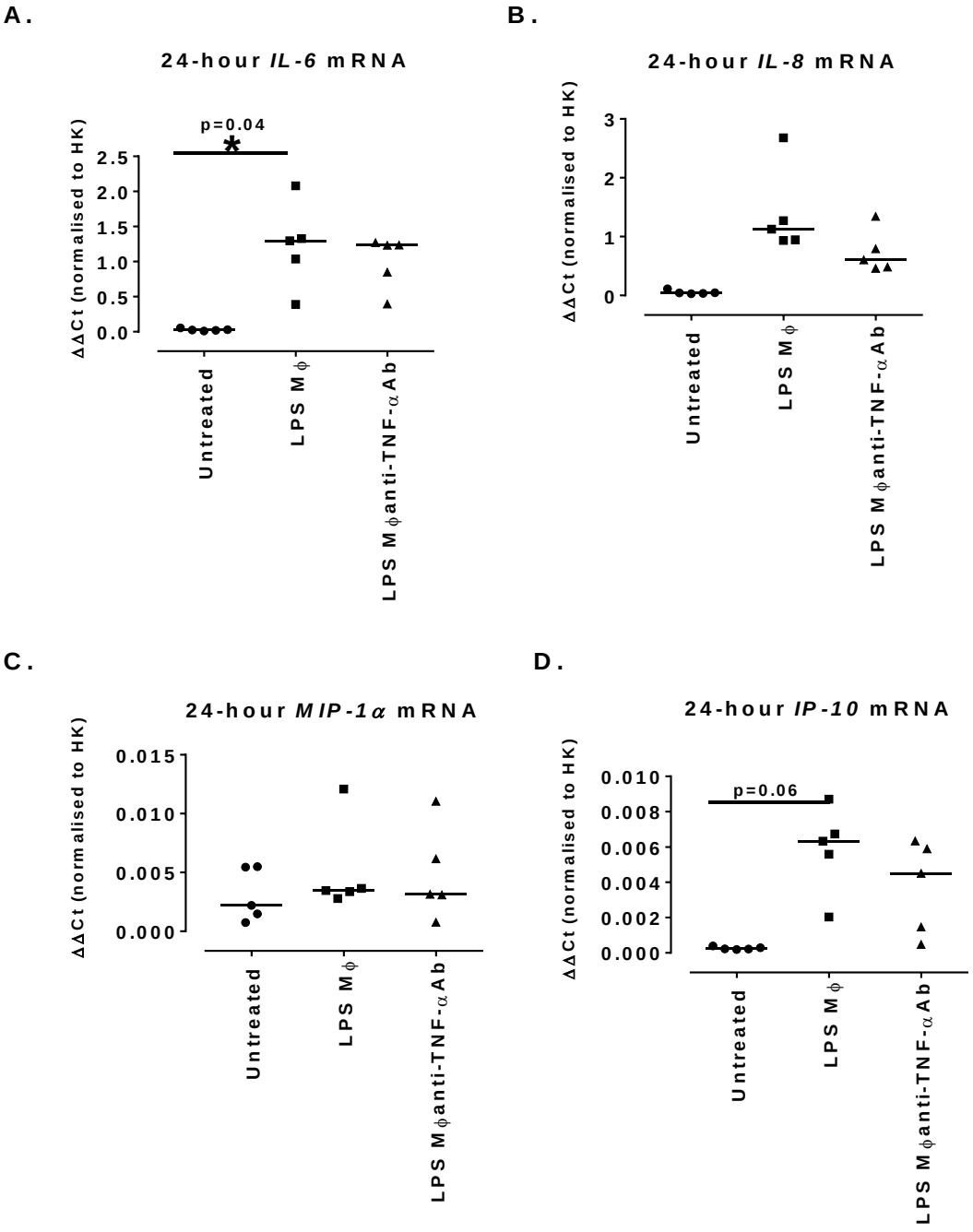

Figure 4.22 Exploring the effect of 24-hour ciGEnC treatment with LPS-treated human macrophage conditioned media -with or without anti-TNF-α Ab addition- on cytokine and chemokine gene expression.

*Changes in IL-6 (A), IL-8 (B), MIP-1α (C) and IP-10 (D) mRNA expression. Data presented as median ΔΔCt [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05 vs LPS MΦ-ciGEnCs. N=5/group. HK: TBP, TUBB.*

4.4.3.2.Urinary biomarker mRNA expression after treatment with MΦ conditioned media

The LPS MΦ media statistically significantly increased *MCP-1* mRNA expression (11 [4-23], p=0.04) compared to untreated ciGEnCs (0.3 [0.2-1.2]) and anti-TNF-αAb (Ab) addition to LPS MΦ media, prior to ciGEnC incubation, partially reduced *MCP-1* mRNA levels (7 [4-10], p=NS) (**Figure 4.23-A**). For *VCAM-1* mRNA expression, no trends or statistically significant changes were observed, when the untreated (0.006 [0.002-0.01]), LPS MΦ-treated (0.08 [0.009-0.2]) and LPS MΦ/anti-TNF-α-treated ciGEnCs (0.5 [0.006-0.07]) were compared (**Figure 4.23-B**).

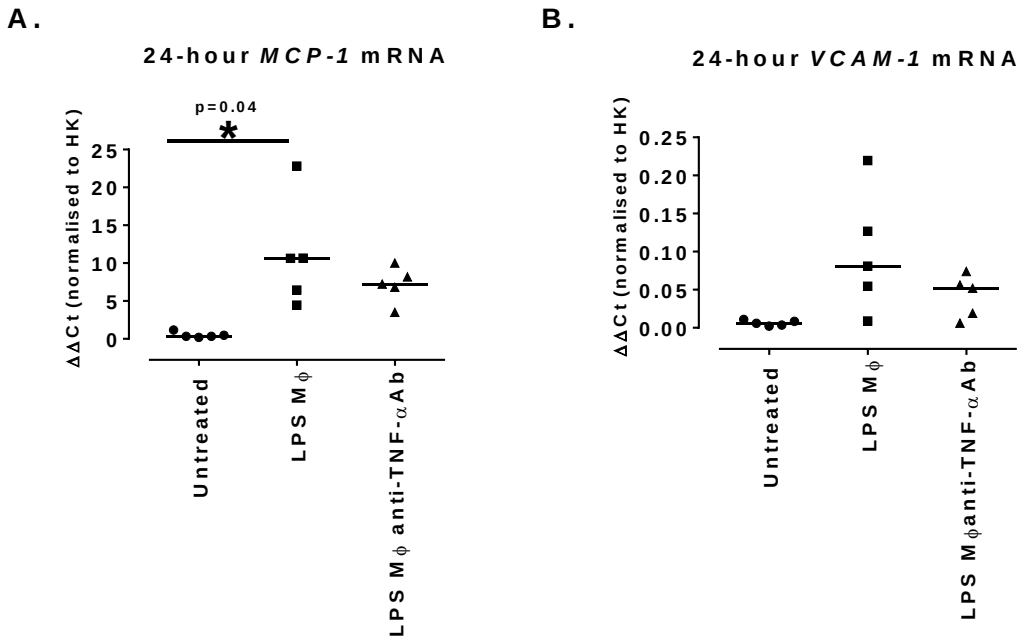

Figure 4.23 Exploring the effect of 24-hour ciGEnC treatment with LPS-treated human macrophage conditioned media -with or without anti-TNF-αAb addition- on urinary biomarker gene expression.

*Changes in MCP-1 (A), VCAM-1 (B) mRNA expression. Data presented as median ΔΔCt [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05 vs LPS MΦ-ciGEnCs. N=5/group. HK: TBP, TUBB.*

4.4.3.3.Blood cell growth factor mRNA expression after treatment with MΦ conditioned media

No trends or statistically significant changes were observed for *M-CSF* mRNA expression, however the LPS MΦ media treatments (0.01 [0.006-0.03]) led to generally higher *M-CSF* mRNA levels compared to untreated ciGEnCs (0.003 [0.001-0.004]) (**Figure 4.24-A**). No remarkable changes were observed for *GM-CSF* mRNA levels (**Figure 4.24-B**).

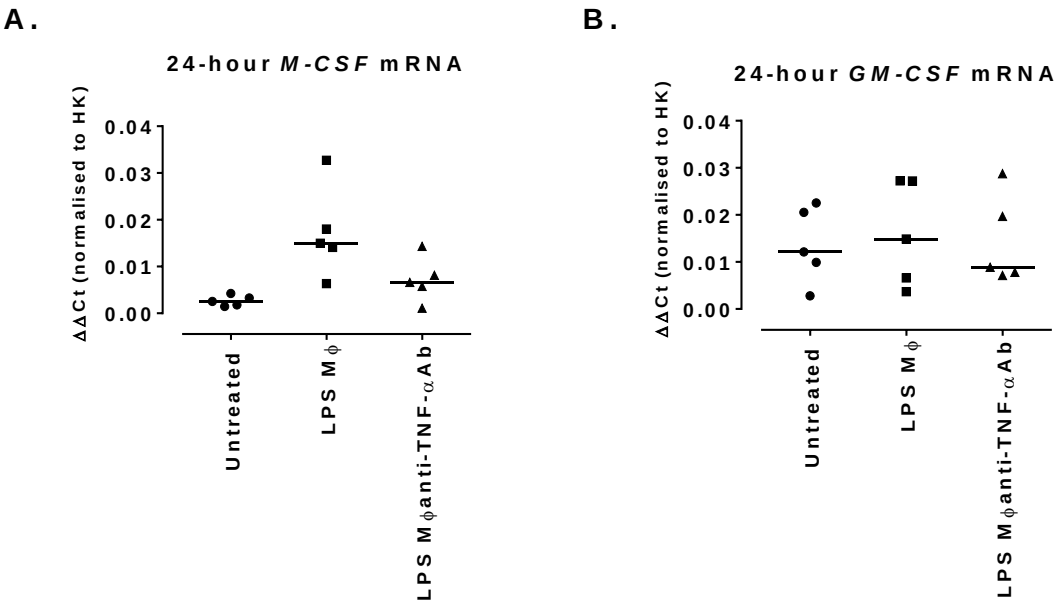

Figure 4.24 Exploring the effect of 24-hour ciGEnC treatment with LPS-treated human macrophage conditioned media -with or without anti-TNF-αAb addition- on blood cell growth factor gene expression.

*Changes in M-CSF (A), GM-CSF (B) mRNA expression. Data presented as median ΔΔCt [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05 vs LPS MΦ-ciGEnCs. N=5/group. HK: TBP, TUBB.*

4.4.3.4.Cytokine receptors mRNA expression after treatment with MΦ conditioned media

A trend was observed for increased *TNFR1* mRNA expression after LPS MΦ treatments (0.1 [0.03-0.1], p=0.07) compared to untreated ciGEnCs (0.05 [0.03-0.06]) (**Figure 4.25-A**). Anti-TNF-α Ab addition to the LPS MΦ media did not reverse the LPS MΦ media effect (0.09 [0.07-0.1]), suggesting that other pro-inflammatory factors secreted by the LPS-treated human macrophages or LPS itself might induce increased *TNFR1* levels (**Figure 4.25-A**). No statistically significant changes were observed for *TNFR2* (**Figure 4.25-B**).

The LPS MΦ media significantly increased *IL-1RI* (0.01 [0.003-0.01], p=0.05) (**Figure 4.25-C)** and *IL-1RII* (0.01 [0.01-0.02], p=0.03) (**Figure 4.25-D)** mRNA expression compared to untreated ciGEnCs (*IL-1RI*: 0.002 [0.001-0.006], *IL-1RII*: 0.004 [0.002-0.007]). Anti-TNF-α Ab addition to the LPS MΦ media did not reverse the LPS MΦ media effect for either *IL-1RI* (0.01 [0.008-0.02]) (**Figure 4.25-C)** or *IL-1RII* (0.01 [0.009-0.02]) (**Figure 4.25-D)**, suggesting that other pro-inflammatory factors secreted by the LPS-treated human macrophages or LPS itself might induce increased *IL-1RI* and *IL-1RII* levels.

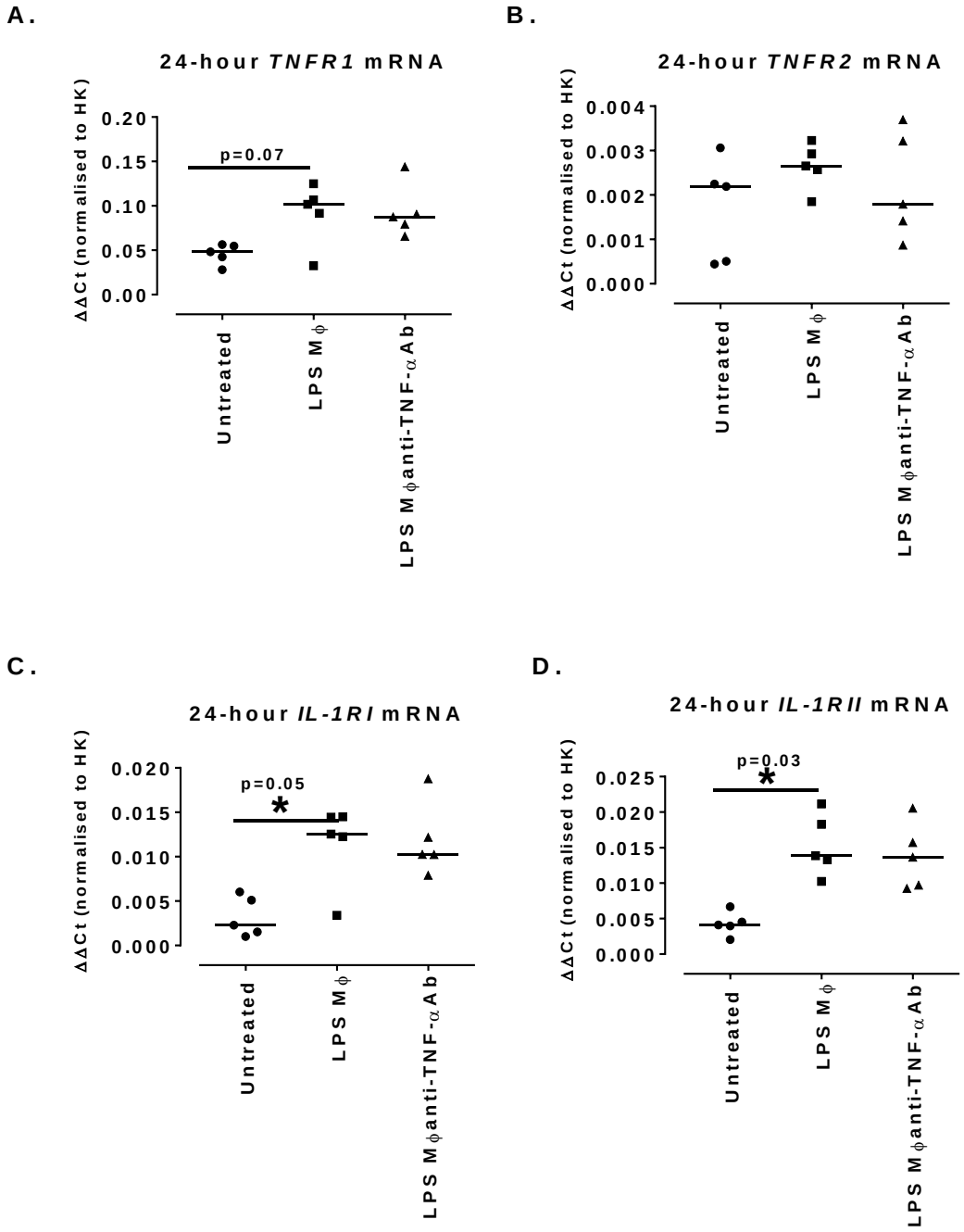

Figure 4.25 Exploring the effect of 24-hour ciGEnC treatment with LPS-treated human macrophage conditioned media -with or without anti-TNF-αAb addition- on TNF-α and IL-1β receptor gene expression.

*Changes in TNFR1 (A), TNFR2 (B), IL-1RI (C), IL-1RII (D) mRNA expression. Data presented as median ΔΔCt [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *p≤0.05 vs LPS MΦ-ciGEnCs. N=5/group. HK: TBP, TUBB.*

4.4.4. Effect of THP-1 conditioned media on pro-inflammatory gene expression by ciGEnCs

To develop a more reproducible *in vitro* model for the study of the potential paracrine effect of human THP-1 macrophages on ciGEnCs, the THP-1 macrophages were initially stimulated for 48h with IFN-γ and LPS, with untreated THP-1 cells always being included in all assays.

The untreated, IFN-γ- and LPS-stimulated THP-1 conditioned media were collected and initially, the presence of TNF-α and IL-1β within the THP-1 conditioned media was tested via ELISA.

4.4.4.1.TNF-α and IL-1β secretion by the THP-1 macrophages

In order to determine which mediators are being secreted from activated THP-1 cells that may be inducing pro-inflammatory changes within GEnCs the secretion of TNF-α and IL-1β by the THP-1 macrophages was tested after 48h of individual IFN-γ and LPS incubations.

TNF-α secretion was significantly increased by LPS treatments (217 pg/ml [143-503], p=) compared to untreated THP-1 cells (11 pg/ml [8-22]) (**Figure 4.26-A**) whereas IFN-γ did not modify basal THP-1 TNF-α secretion levels (11 pg/ml [3-33]) (**Figure 4.26-A**). IFN-γ (67 pg/ml [37-135]) and LPS (72 pg/ml [50-228]) treatments did not significantly increase basal THP-1 IL-1β levels when compared to untreated ciGEnCs (32 pg/ml [29-101]) (**Figure 4.26-B**).

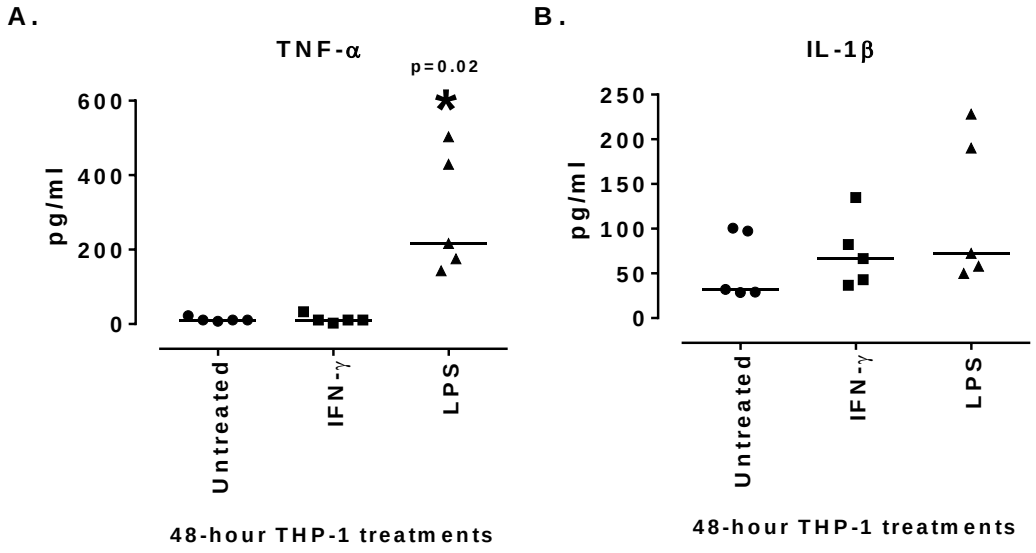

Figure 4.26 Changes in TNF-α and IL-1β secretion levels and fold changes following 48-hour stimulation of THP-1 cells with IFN-γ and LPS.

*Changes in TNF-α (A), IL-1β (B) secretion levels. Data presented as median concentration [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *p≤0.005, N=5/group.*

4.4.4.2.Investigating the effect of THP-1 conditioned media on ciGEnC expression and/or secretion of pro-inflammatory proteins

Once it had been determined that TNF-α and IL-1β are expressed by THP-1 cells it was hypothesised that these cytokines could be responsible for inducing pro-inflammatory gene expression by ciGEnCs in response to THP-1 conditioned media treatments.

The untreated, IFN-γ- and LPS-stimulated THP-1 conditioned media were collected and were used with or without incubation with anti-TNF-α Ab and IL-1Ra for 2 hours at 37^{o}C and 5% CO_2 to stimulate the ciGEnCs for 24h. Subsequently, the expression and/or secretion of pro-inflammatory mediators were assessed via qPCR and ELISA assays.

4.4.4.3. Cytokine and chemokine mRNA expression after treatment with THP-1 conditioned media

ciGEnCs expressed low levels of mRNA for *IL-6* (**Figure 4.27-A**) under basal conditions (untreated ciGEnCs), and no significant change was noted following incubation with conditioned media from untreated THP-1 cells. When treated with conditioned media from IFN-γ- and LPS-activated THP-1 cells the ciGEnC expression levels of *IL-6* did not differ from those of the IFN-γ- and LPS-treated ciGEnCs. (**Figure 4.27-A**).

ciGEnC *IL-8* mRNA levels were statistically significantly lower when anti-TNF-α Ab/IL-1Ra were added to the untreated THP-1 media (0.07 [0.05-0.2]) compared to the untreated THP-1 media (0.4 [0.3-0.6], p=0.01) alone (**Figure 4.27-B**). Nevertheless, the untreated THP-1 media-induced *IL-8* levels did not significantly differ from the untreated ciGEnCs' basal levels (0.2 [0.1-0.7]), although they were slightly higher (**Figure 4.27-B**). The IFN-γ THP-1 media significantly increased *IL-8* mRNA expression (0.7 [0.1-7], p=0.018); this could not be attributed to the effects of residual IFN-γ as direct stimulation of ciGEnCs with IFN-γ did not replicate this effect (**Figure 4.27-B**). Anti-TNF-α Ab/IL-1Ra addition brought *IL-8* levels (0.07 [0.02-2]) closer to those of the IFN-γ-treated ciGEnCs (**Figure 4.27-B**).

ciGEnCs expressed low levels of mRNA for *MIP-1α* (**Figure 4.27-C**) and *IP-10* (**Figure 4.27-D**) under basal conditions (untreated ciGEnCs), and no significant change was observed after stimulation with conditioned media from untreated THP-1 cells. When treated with conditioned media from IFN-γ- and LPS-activated THP-1 cells the ciGEnC expression levels of *MIP-1α* and *IP-10* did not differ from those of the IFN-γ- and LPS-treated ciGEnCs.

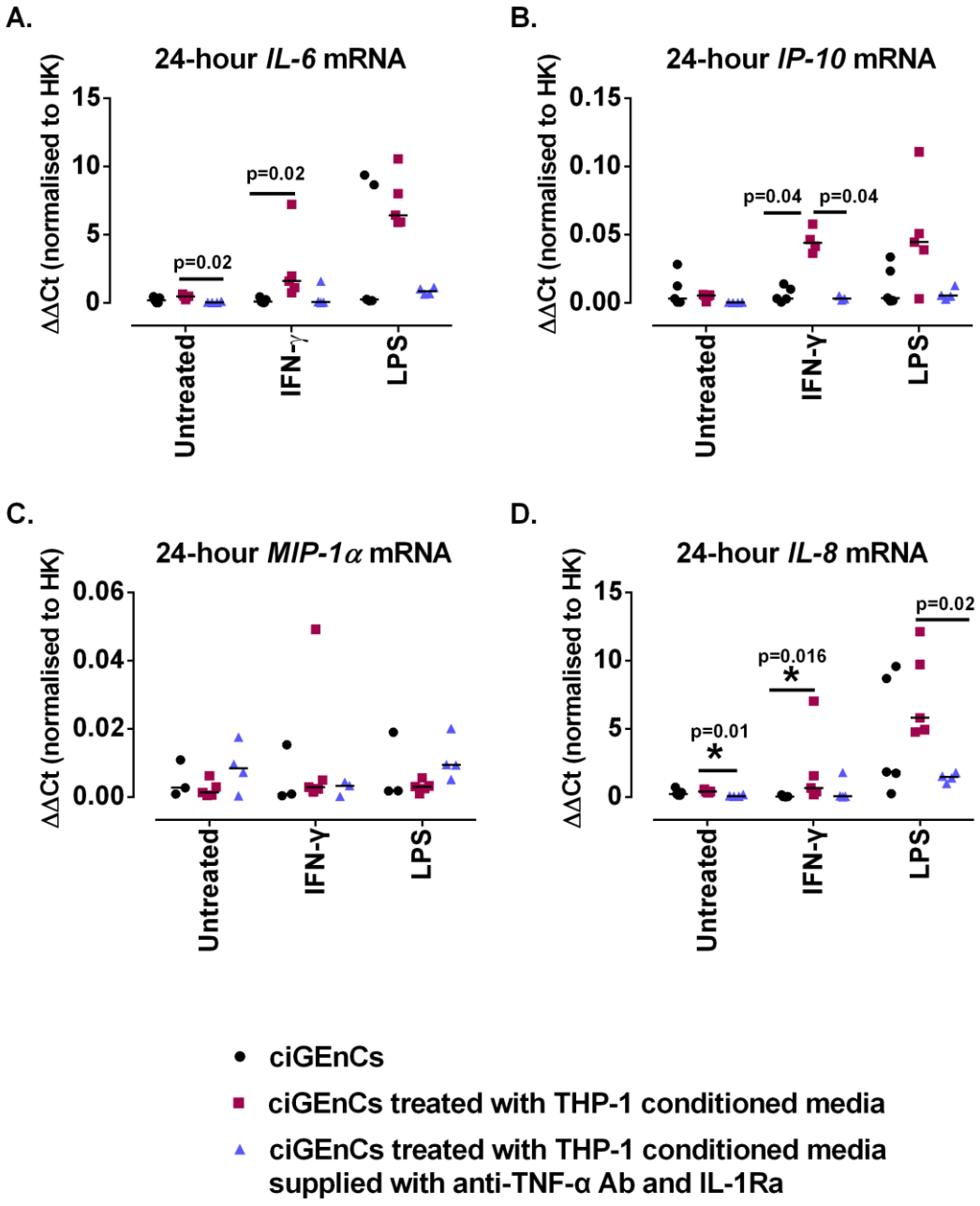

Figure 4.27 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media, with or without anti-TNF-α Ab/IL-1Ra addition, on cytokine and chemokine mRNA expression.

*Changes in IL-6 (A), IL-8 (B), MIP-1α (C), IP-10 (D) mRNA expression. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). *p≤0.017, N=3-5/group. HK: YWHAZ.*

4.4.4.4.Urinary biomarker mRNA expression after treatment with THP-1 conditioned media

No statistically significant changes were observed in *MCP-1* mRNA expression which was lowly expressed by untreated ciGEnCs and these expression levels were not statistically significantly modified either by untreated THP-1 conditioned media or by the rest of the IFN-γ-/LPS-stimulated THP-1 conditioned media and IFN-γ/LPS ciGEnCs treatments (**Figure 4.28-A**).

The IFN-γ THP-1 media led to statistically significantly higher *VCAM-1* mRNA expression levels (0.02 [0.01-0.8], p=0.059) compared to the IFN-γ THP-1 media with anti-TNF-α/IL-1Ra addition (0.002 [0.0008-0.007]) (**Figure 4.28-B**). The IFN-γ THP-1 media did not lead to statistically significantly higher *VCAM-1* mRNA expression when compared to IFN-γ-treated ciGEnCs (0.01 [0.006-0.02]), although the *VCAM-1* mRNA levels of the latter were lower than those of the IFN-γ THP-1 media-treated ciGEnCs (**Figure 4.28-B**).

Similar to the IFN-γ THP-1 media, the LPS THP-1 media led to statistically significantly higher *VCAM-1* mRNA expression levels (0.05 [0.04-0.2], p=0.01) compared to the LPS THP-1 media with anti-TNF-α/IL-1Ra addition (0.007 [0.005-0.02]) (**Figure 4.28-B**). The LPS THP-1 media did not lead to statistically significantly higher *VCAM-1* mRNA expression when compared to LPS-treated ciGEnCs (0.01 [0.01-0.1]), although the *VCAM-1* mRNA levels of the latter were lower than those of the (LPS THP-1 media)-treated ciGEnCs (**Figure 4.28-B**).

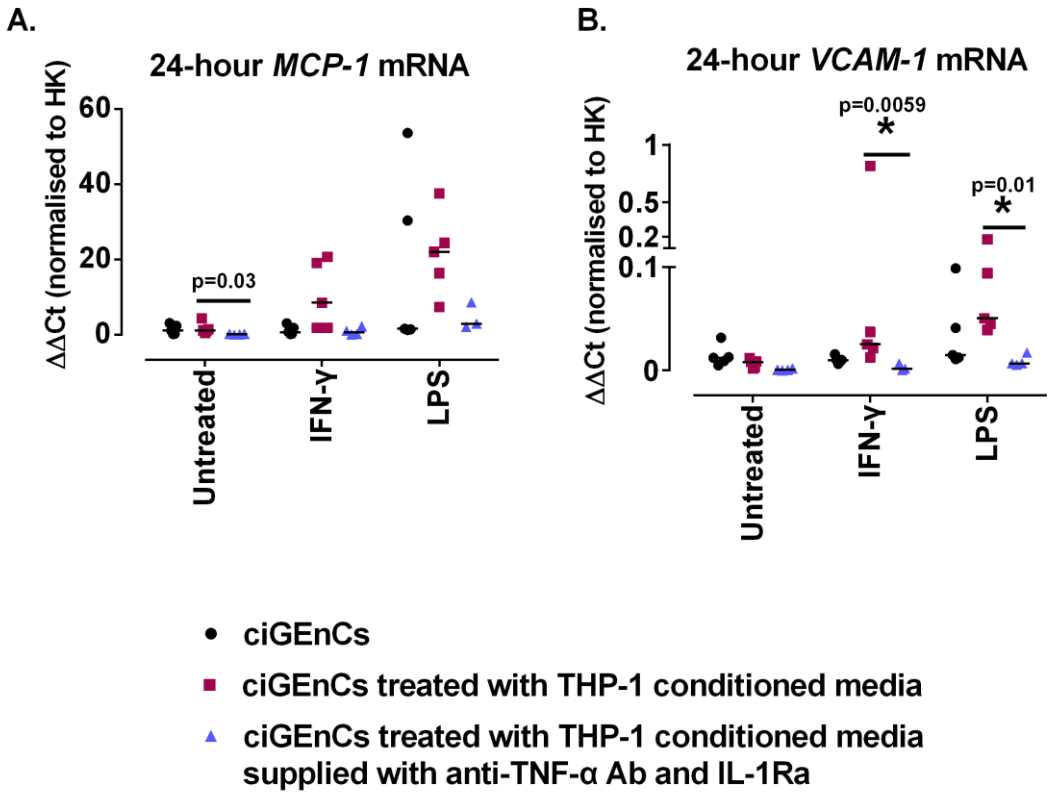

Figure 4.28 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media, with or without anti-TNF-α Ab/IL-1Ra addition, on urinary biomarker gene expression.

*Changes in MCP-1 (A), VCAM-1 (B) mRNA expression. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). *p≤0.017, N=3-5/group. HK: YWHAZ.*

4.4.4.5.Blood cell growth factor mRNA expression after treatment with THP-1 conditioned media

No statistically significant changes were observed in *M-CSF* (**Figure 4.29-A**) and *GM-CSF* (**Figure 4.29-B**) levels of mRNA expression which were lowly expressed by untreated ciGEnCs. These basal expression levels were not statistically significantly modified either by untreated THP-1 conditioned media or by the rest of the IFN-γ-/LPS-stimulated THP-1 conditioned media and IFN-γ/LPS ciGEnCs treatments (**Figure 4.29**).

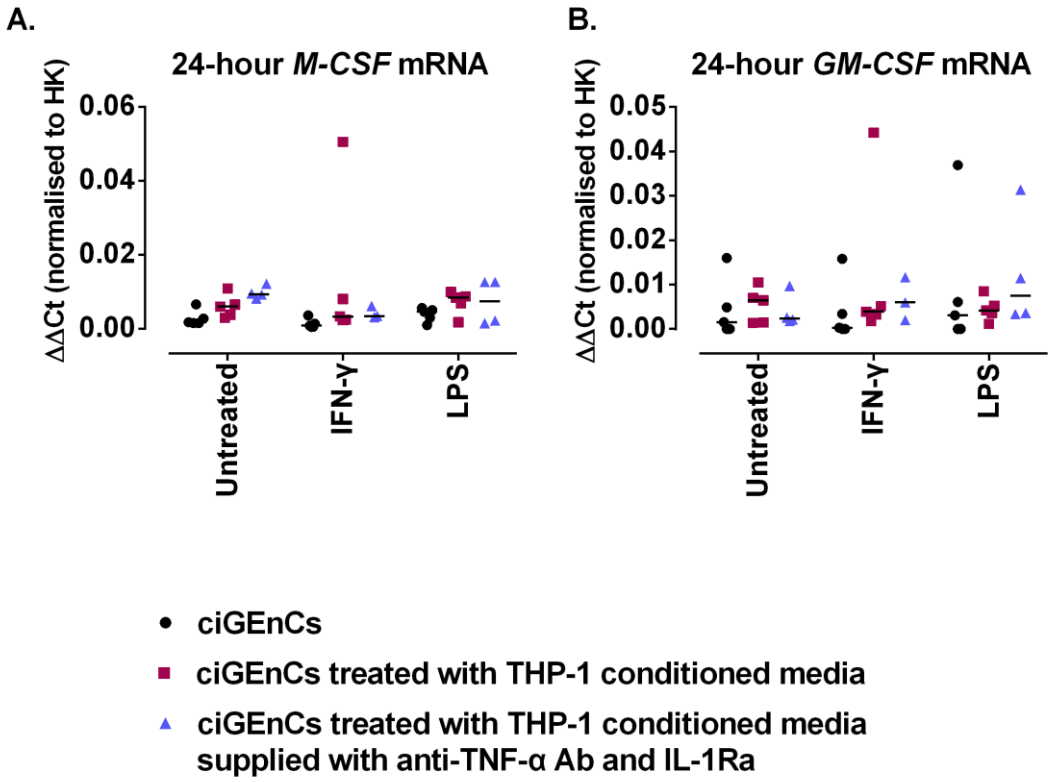

Figure 4.29 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media, with or without anti-TNF-αAb/IL-1Ra addition, on blood cell growth factor gene expression.

Changes in M-CSF (A), GM-CSF (B) mRNA expression. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). p=NS, N=3-5/group. HK: YWHAZ.

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4.4.4.6.Cytokine receptor mRNA expression after treatment with THP-1 conditioned media

No statistically significant differences were observed for *TNFR1* mRNA expression (**Figure 4.30-A**). Anti-TNF-α Ab/IL-1Ra addition to the untreated THP-1 ($3x10^{-10}$ [$2x10^{-10}$ -$6x10^{-10}$]), IFN-γ THP-1 (0.005 [0.003-0.04]) and LPS THP-1 media ($2x10^{-10}$ [$4x10^{-11}$ -$3x10^{-10}$]) prior to ciGEnC incubation led to a statistically significant decrease in *TNFR2* expression when compared to ciGEnCs treated with untreated THP-1 media (0.003 [0.0004-0.004], p=0.017), IFN-γ THP-1 media (0.005 [0.003-0.04], p=0.014) and

LPS THP-1 media (0.007 [0.005-0.01], p=0.014) respectively (**Figure 4.30-B**). No significant changes were observed when untreated ciGEnCs (0.002 [0.001-0.003]), IFN-γ-treated (0.001 [0.0007-0.002]) and LPS-treated ciGEnCs (0.002 [0.001-0.004]) were compared to their THP-1 media counterparts, although their *TNFR2* expression was generally lower.

No statistically significant differences were observed for *IL-1RI* mRNA expression (**Figure 4.30-C**). Anti-TNF-α Ab/IL-1Ra addition to the untreated THP-1 (0.0007 [0.0004-0.001]), IFN-γ THP-1 (0.0009 [0.0006-0.001]) and LPS THP-1 media (0.0004 [0.0002-0.008]) prior to ciGEnC incubation, led to a statistically significant decrease in *IL-1RII* expression when compared to ciGEnCs treated with untreated THP-1media (0.007 [0.004-0.03], p=0.017), IFN-γ THP-1 media (0.008 [0.003-0.03], p=0.014) and LPS THP-1 media (0.007 [0.005-0.01], p=0.014) respectively (**Figure 4.30-D**). No significant changes were observed when untreated ciGEnCs (0.005 [0.003-0.01]), IFN-γ-treated (0.006 [0.003-0.008]) and LPS-treated ciGEnCs (0.009 [0.003-0.02]) were compared to their THP-1 media counterparts, although their *IL-1RII* expression was generally lower.

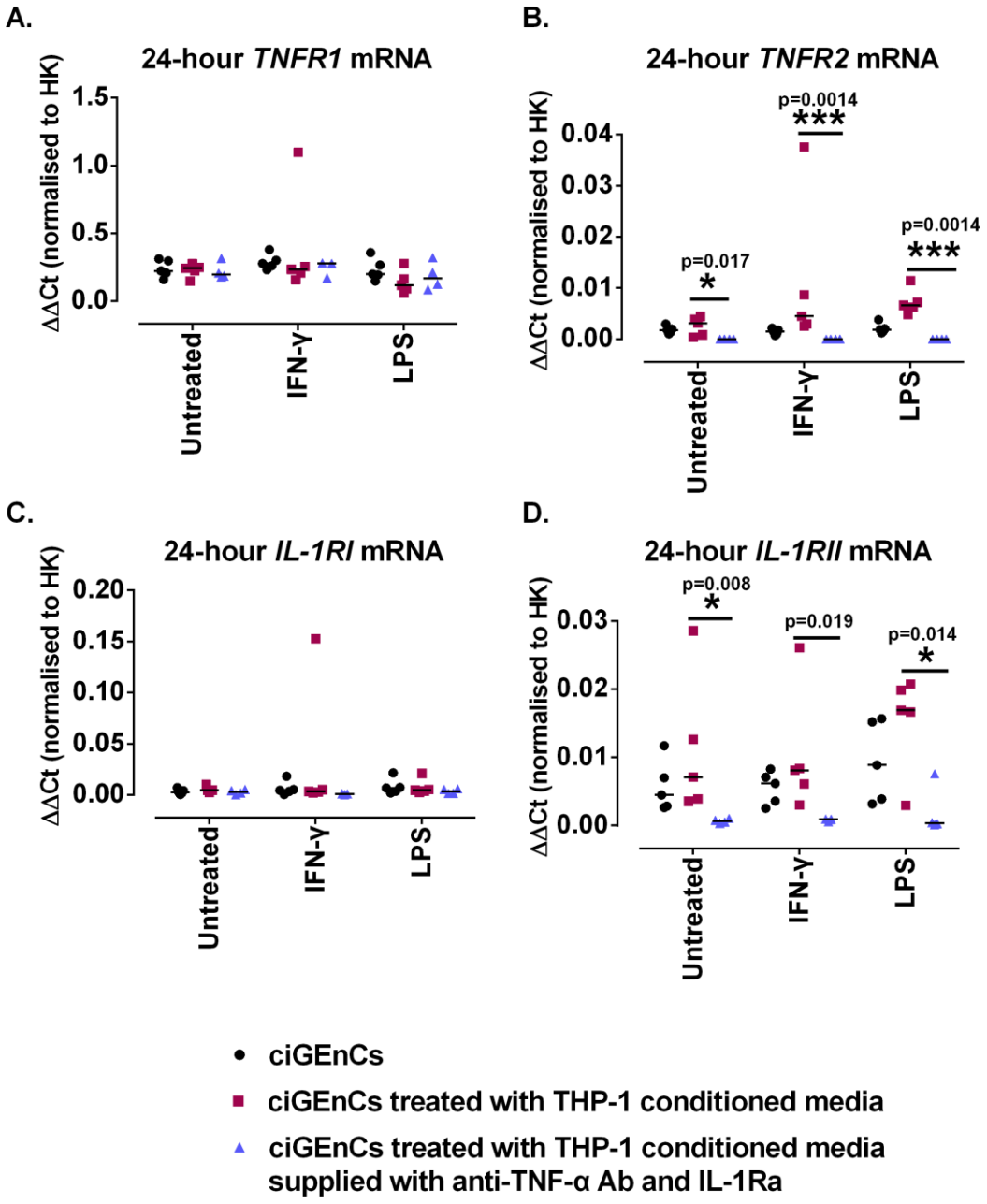


Figure 4.30 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media -with or without anti-TNF-α Ab/IL-1Ra addition- on TNF-α and IL-1β receptor gene expression.

*Changes in TNFR1 (A), TNFR2 (B), IL-1RI (C), IL-1RII (D) mRNA expression. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). *p≤0.017, ***p≤0.0019, N=3-5/group. HK: YWHAZ.*

4.4.4.7. Cytokine and chemokine secretion after treatment with THP-1 conditioned media

The untreated THP-1 media induced a statistically significant increase in IL-6 secretion (3,910 pg/ml [2,688-6,431], p=0.004) compared to untreated ciGEnCs (1,805 pg/ml [1,246-1,949]) (**Figure 4.31-A**). Addition of anti-TNF-α Ab/IL-1Ra to the THP-1 media prior to ciGEnC incubation led to generally lower IL-6 levels (1,954 pg/ml [1,768-2,275]) compared to the untreated THP-1 media effect, although not statistically significantly different (**Figure 4.31-A**).

The IFN-γ THP-1 media induced a statistically significant increase in IL-8 secretion (6,336 pg/ml [6,169-6,507], p=0.002) compared to IFN-γ-treated ciGEnCs (1,328 pg/ml [981-3,004]) (**Figure 4.31-B**). Addition of anti-TNF-α Ab/IL-1Ra to the THP-1 media prior to ciGEnC incubation did not sufficiently reduce the IL-8 levels (5,550 pg/ml [4,916-6,148]) suggesting that factors present within the IFN-γ THP-1 media other than TNF-α and IL-1β could have a more robust effect on the upregulation of IL-8 levels (**Figure 4.31-B**).

Finally, the untreated THP-1 media induced a statistically significant increase in IP-10 secretion (389 pg/ml [156-2,223], p=0.008) compared to untreated ciGEnCs (5pg/ml [5-6]) (**Figure 4.31-C**). Addition of anti-TNF-α Ab/IL-1Ra to the THP-1 media prior to ciGEnC incubation did not lead to statistically significantly lower levels of IP-10 (187 pg/ml [111-692]) compared to the untreated THP-1 media effect (**Figure 4.31-C**).

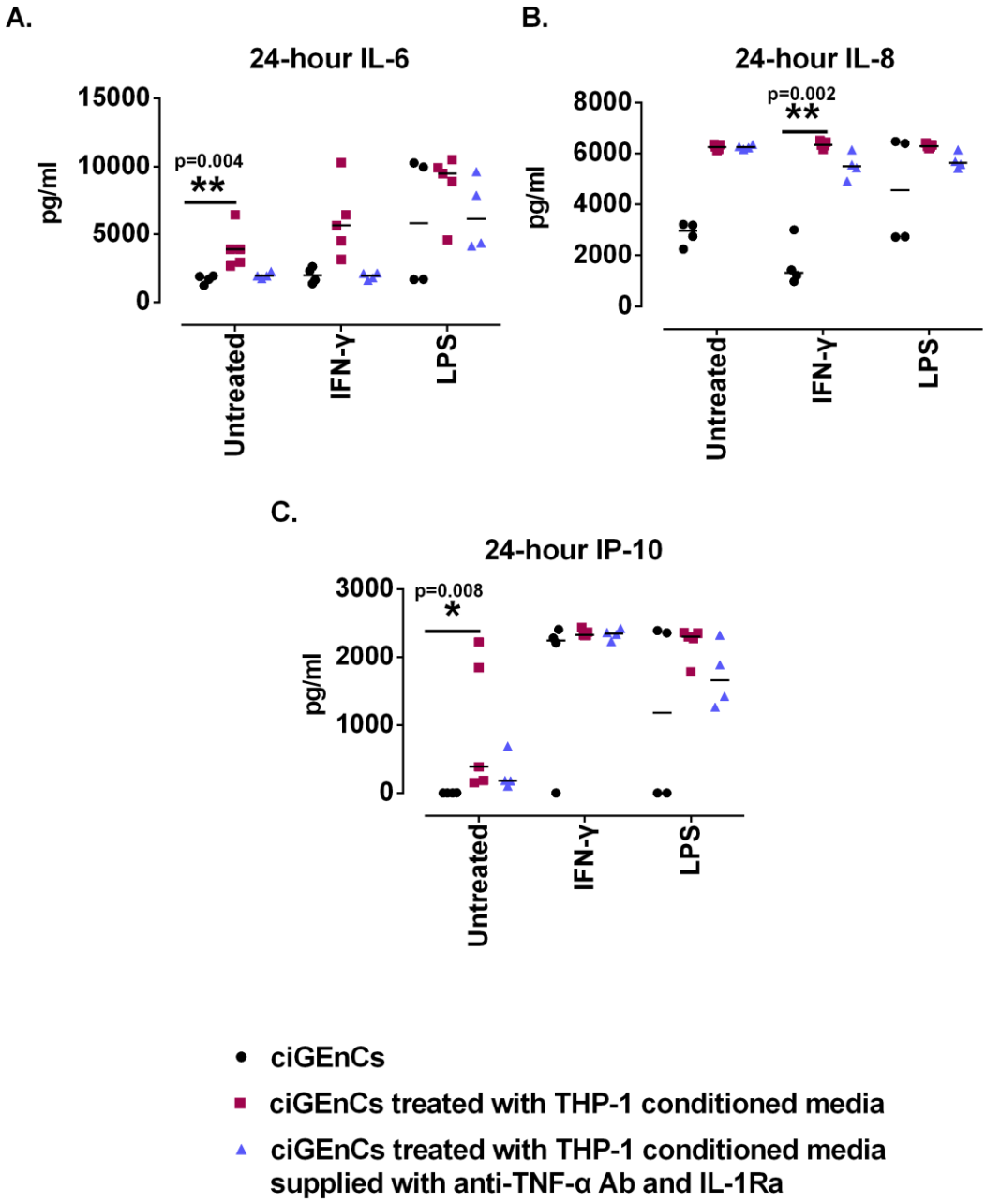


Figure 4.31 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media -with or without anti-TNF-αAb/IL-1Ra addition- on cytokine and chemokine secretion.

*Changes in IL-6 (A), IL-8 (B), IP-10 (C) secretion. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). *p≤0.017, **p≤0.0057, N=4-5/group.*

4.4.4.8.Urinary biomarker protein secretion after treatment with THP-1 conditioned media

The IFN-γ THP-1 media significantly increased MCP-1 secretion (1,252 pg/ml [1,128-1,501], p=0.008) compared to IFN-γ-treated ciGEnCs (255 pg/ml [235-292]) (**Figure 4.32-A**). Addition of anti-TNF-α Ab/IL-1Ra to the THP-1 media prior to ciGEnC incubation, led to generally lower MCP-1 levels (491 pg/ml [218-557]) compared to the IFN-γ THP-1 media effect, although no statistically significant differences were observed (**Figure 4.32-A**).

sVCAM-1 secretion following IFN-γ THP-1 media treatments (131 pg/ml [29-187], p=0.071) was statistically significantly increased compared to IFN-γ THP-1 media with anti-TNF-α Ab/IL-1Ra addition (8 pg/ml [0-20]) (**Figure 4.32-B**). The IFN-γ THP-1 media induced generally higher-but not statistically significantly higher- sVCAM-1 levels compared to IFN-γ-treated ciGEnCs (64 pg/ml [59-70]) (**Figure 4.32-B**).

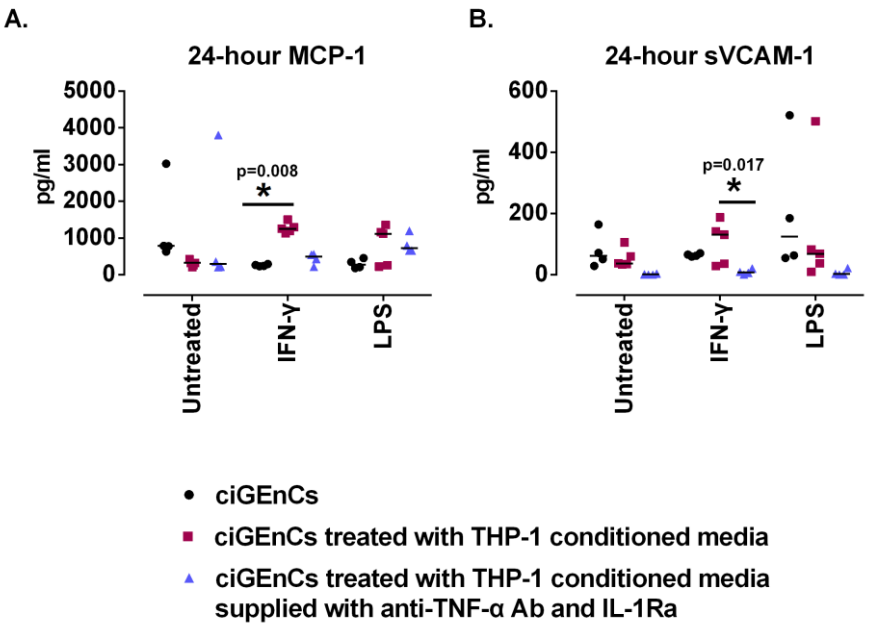


Figure 4.32 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media -with or without anti-TNF-αAb/IL-1Ra addition- on urinary biomarker secretion.

*Changes in MCP-1 (A), sVCAM-1 (B) secretion. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). *p≤0.017, N=4-5/group.*

4.4.4.9. Blood cell growth factor secretion after treatment with THP-1 conditioned media

The untreated THP-1 media significantly increased M-CSF secretion (1,527 pg/ml [1,161-1,907], p=0.013) compared to the untreated ciGEnCs (570 pg/ml [321-672]) (**Figure 4.33-A**). Nevertheless, addition of anti-TNF-α Ab/IL-1Ra did not manage to sufficiently suppress M-CSF secretion (1,224 pg/ml [1,171-1,832]) suggesting that other factors than TNF-α and IL-1β, present within the untreated THP-1 media, could also play a major role in M-CSF secretion (**Figure 4.33-A**).

The untreated THP-1 media (1,036 pg/ml [777-1,335], p=0.002) and the IFN-γ THP-1 media (1,020 pg/ml [457-3,847], p=0.002) significantly increased GM-CSF secretion compared to the untreated (176 pg/ml [102-187]) and IFN-γ-treated ciGEnCs (69 pg/ml [33-188]) respectively (**Figure 4.33-B**). Addition of anti-TNF-α/IL-1Ra did not statistically significantly suppress the effect of the untreated THP-1 media (406 pg/ml [264-534]) and of the IFN-γ THP-1 media (228 pg/ml [195-264]) on GM-CSF levels (**Figure 4.33-B**).

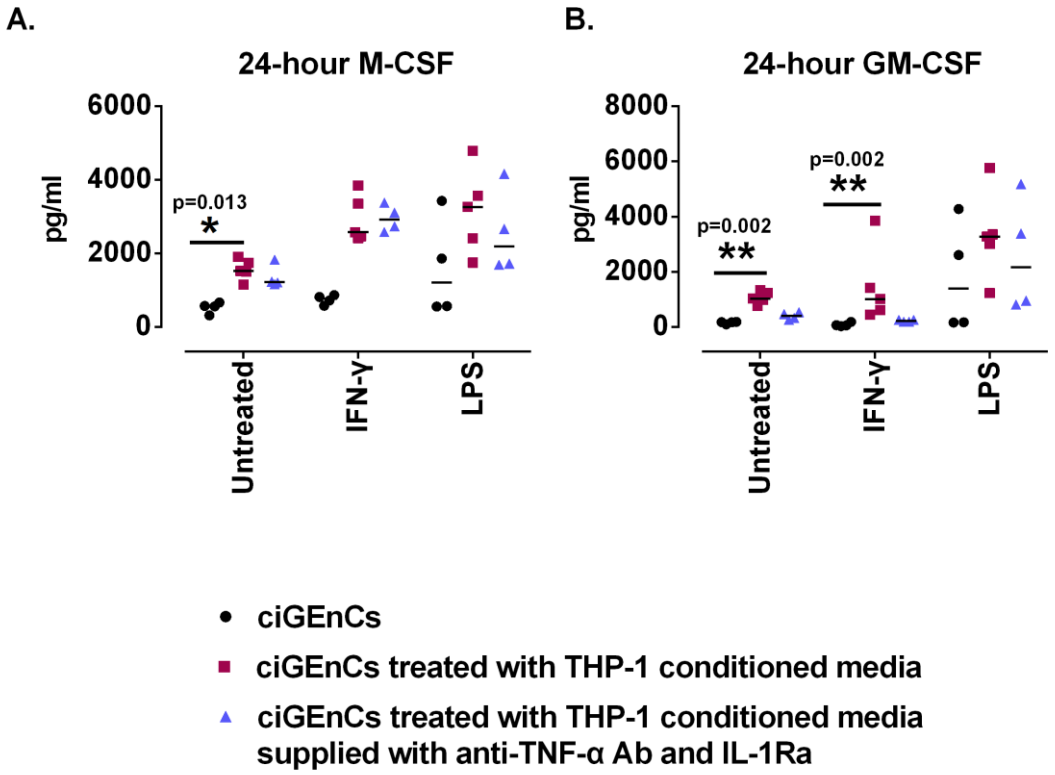


Figure 4.33 Effect of 24-hour ciGEnC treatment with untreated, IFN-γ- and LPS-treated THP-1 conditioned media -with or without anti-TNF-α Ab/IL-1Ra addition- on blood cell growth factor secretion.

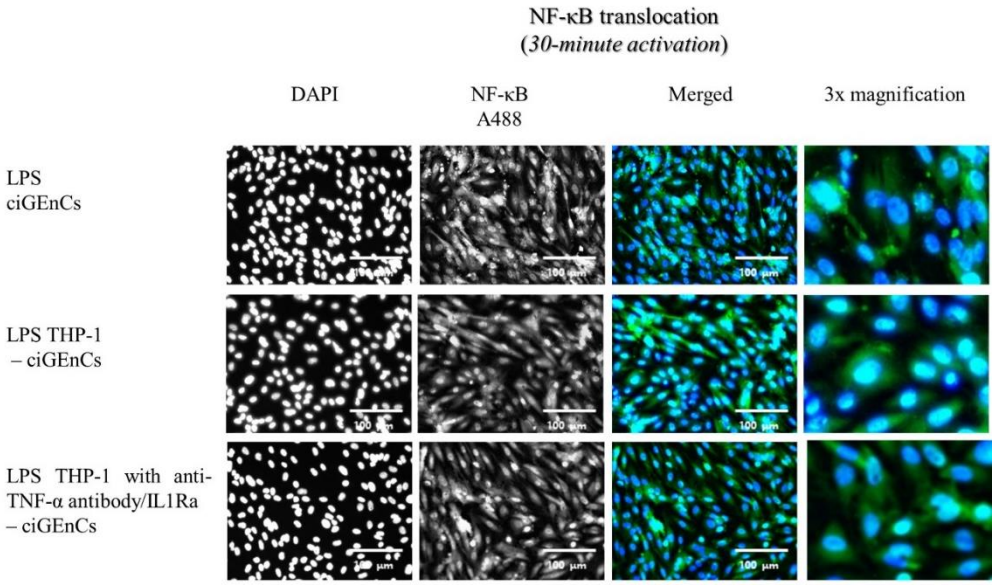
*Changes in M-CSF (A), GM-CSF (B) secretion. Data presented as interleaved scatter plots are analysed with Kruskal-Wallis with Dunn's post-hoc test with corrected p-values for each treatment group (ciGEnCs vs THP-1 conditioned media-treated ciGEnCs: Untreated, IFN-γ, LPS). *p≤0.017, **p≤0.0057 N=4-5/group.*

4.4.4.10. NF-κB activation by LPS THP-1 media

As the LPS-stimulated THP-1 conditioned media demonstrated the highest levels and fold-increases of TNF-α and IL-1β secretion (**Figure 4.26**), their effect on ciGEnC NF-κB activation was tested via IF (**Figure 4.34**). The ciGEnCs were treated for 30 minutes with LPS alone, LPS THP-1 media and LPS THP-1 media with anti-TNF-α/IL-1Ra addition and NF-κB nuclear translocation was determined via fluorescent microscopy (**Figure 4.34-A**).

LPS alone, induced a moderate NF-κB nuclear translocation as demonstrated by the r-values for nuclear co-localisation of DAPI and A488 (r-value: 0.67 [0.2-0.9]) whereas incubation of ciGEnCs with LPS THP-1 media significantly increased NF-κB nuclear translocation (r-value: 0.8 [0.1-1], $p<0.0001$) compared to the LPS-treated ciGEnCs (**Figure 4.34-B**). Addition of anti-TNF-α Ab/IL-1Ra to the LPS THP-1 media statistically significantly decreased NF-κB nuclear translocation levels (r-value: 0.7 [0.09-0.9], $p=0.0007$) compared to the LPS THP-1 media-treated ciGEnCs, although they remained relatively high (**Figure 4.34-B**).

A.



B.

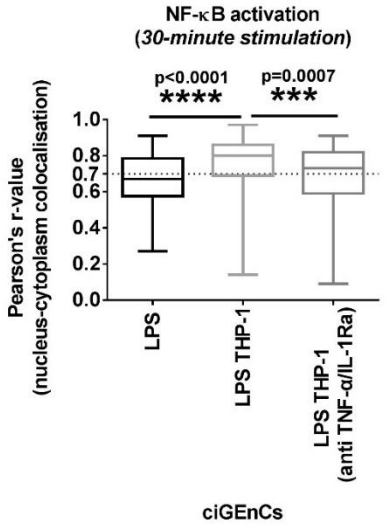


Figure 4.34 Effect of 30-minute ciGEnC treatments with LPS, LPS THP-1 conditioned media and LPS THP-1 conditioned with anti-TNF-α Ab/IL-1Ra addition on NF-κB nuclear translocation.

*A. 20x IF images of ciGEnCs following 30-minute. B. Statistical analysis diagram. Data (r-values for nuclear co-localisation of DAPI and A488 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and are analysed using Kruskal-Wallis test with Dunn's post-hoc test. ***p<0.001, ****p≤0.0001 vs LPS THP-1 conditioned media-treated ciGEnCs. Scale bars: 100μm.*

4.4.5. Effect of podocyte conditioned media on pro-inflammatory gene expression by GEnCs

Conditionally immortalised human podocytes were treated for 24h with pro-inflammatory cytokines -TNF-α, IL-1β, IFN-α, IFN-γ- and LPS. Subsequently, the 24h podocyte conditioned media (together with untreated podocyte conditioned media) were collected and were used to treat the ciGEnCs for 24h. Changes in ciGEnC pro-inflammatory gene expression were then tested.

4.4.5.1.Cytokine mRNA expression after treatment with podocyte conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS podocyte conditioned media were not found to statistically significantly promote or upregulate cytokine mRNA expression (**Figure 4.35**).

The untreated ciGEnCs' basal expression levels of *IL-6* (**Figure 4.35-A**) and *IL-10* (**Figure 4.35-B**) were low and were not modified by the untreated podocyte media treatments. Similarly, no remarkable changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

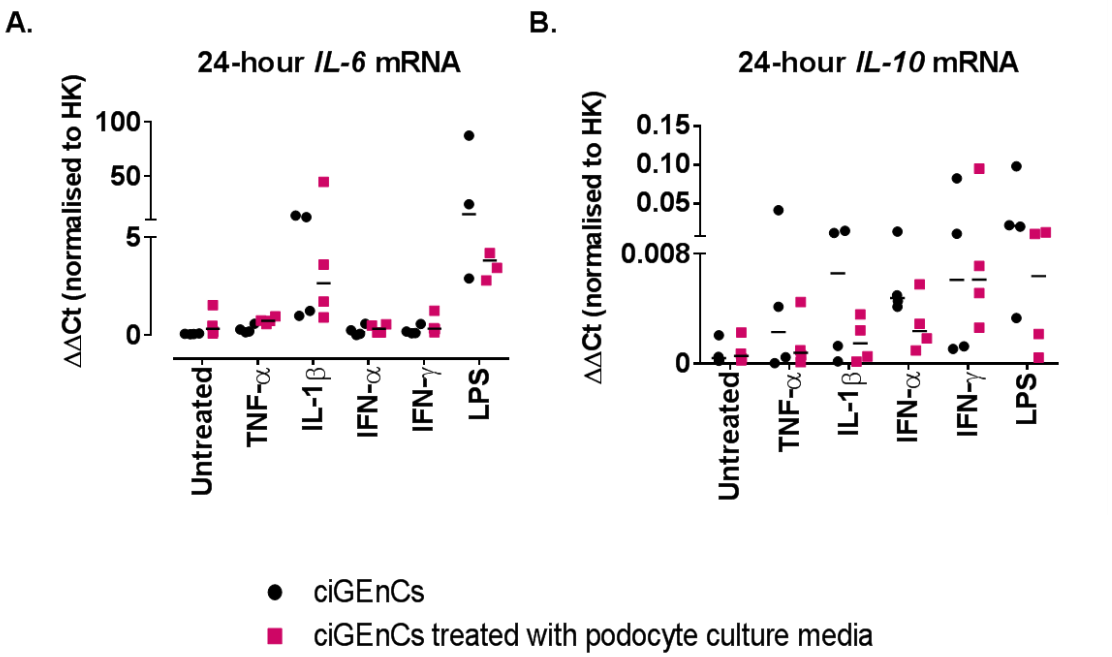


Figure 4.35 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated podocyte conditioned media on cytokine gene expression.

Changes in IL-6 (A), IL-10 (B) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs podocyte conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

4.4.5.2.Chemokine mRNA expression after treatment with podocyte conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS podocyte conditioned media were not found to statistically significantly promote or upregulate chemokine mRNA expression (**Figure 4.36**).

The untreated ciGEnCs' basal expression levels of *IL-8* (**Figure 4.36-A**), *MIP-1α* (**Figure 4.36-B**) and *IP-10* (**Figure 4.36-C**) were low and were not modified by the untreated podocyte media treatments. Similarly, no remarkable changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

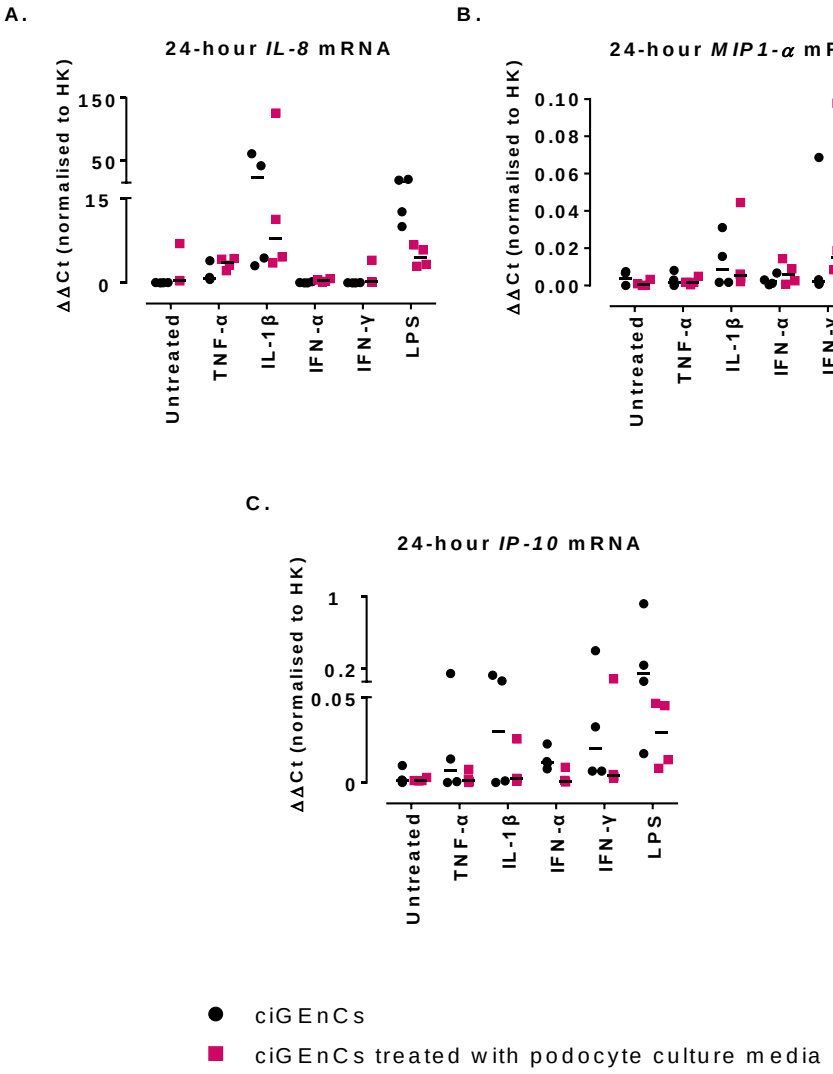


Figure 4.36 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated podocyte conditioned media on chemokine gene expression.

Changes in IL-8 (A), MIP-1α (B), IP-10 (C) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs podocyte conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

4.4.5.3.Urinary biomarker mRNA expression after treatment with podocyte conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS podocyte conditioned media were not found to statistically significantly promote or upregulate urinary biomarker mRNA expression (**Figure 4.37**).

The untreated ciGEnCs' basal expression levels of *MCP-1* (**Figure 4.37-A**) and *VCAM-1* (**Figure 4.37-B**) were low and were not modified by the untreated podocyte media treatments. Similarly, no remarkable changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

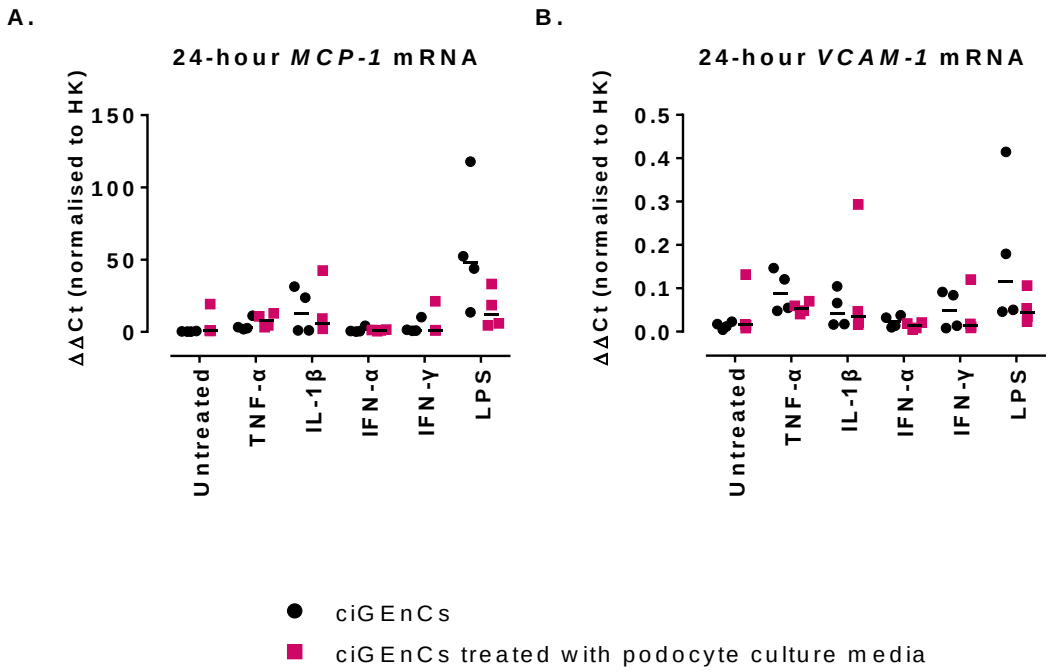


Figure 4.37 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated podocyte conditioned media on urinary biomarker gene expression.

Changes in MCP-1 (A), VCAM-1 (B) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs podocyte conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

4.4.5.4.Blood cell growth factor mRNA expression after treatment with podocyte conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS podocyte conditioned media were not found to statistically significantly promote or upregulate blood cell growth factor mRNA expression (**Figure 4.38**).

The untreated ciGEnCs' basal expression levels of *M-CSF* (**Figure 4.38-A**) and *GM-CSF* (**Figure 4.38-B**) were low and were not modified by the untreated podocyte media treatments. Similarly, no remarkable changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

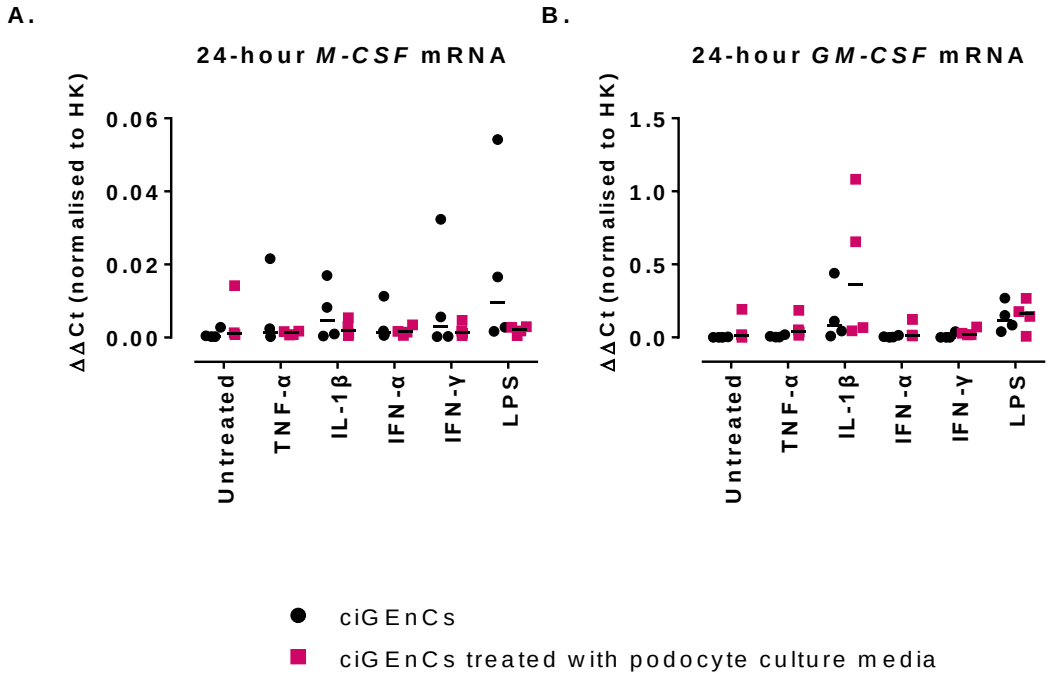


Figure 4.38 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated podocyte conditioned media on blood cell growth factor gene expression.

Changes in M-CSF (A), GM-CSF (B) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs podocyte conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

These findings could suggest that, at this (24h) time-point, the podocytes are unable to contribute to the pro-inflammatory phenotype of the GEnCs potentially due to inadequate pro-inflammatory protein production.

4.4.6. Effect of MC conditioned media on pro-inflammatory gene expression by GEnCs

Conditionally immortalised human MCs were treated for 24h with pro-inflammatory cytokines -TNF-α, IL-1β, IFN-α, IFN-γ- and LPS. Subsequently, the 24h MC conditioned media (together with untreated MC conditioned media) were collected and were used to treat the ciGEnCs for 24h. Changes in ciGEnC pro-inflammatory gene expression were then tested.

4.4.6.1.Cytokine mRNA expression after treatment with MCs conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS MCs conditioned media were not found to statistically significantly increase cytokine mRNA expression (**Figure 4.39**).

The untreated ciGEnCs' basal expression levels of *IL-6* (**Figure 4.39-A**) and *IL-10* (**Figure 4.39-B**) were low and were not altered by the untreated MC media treatments. Similarly, no robust changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

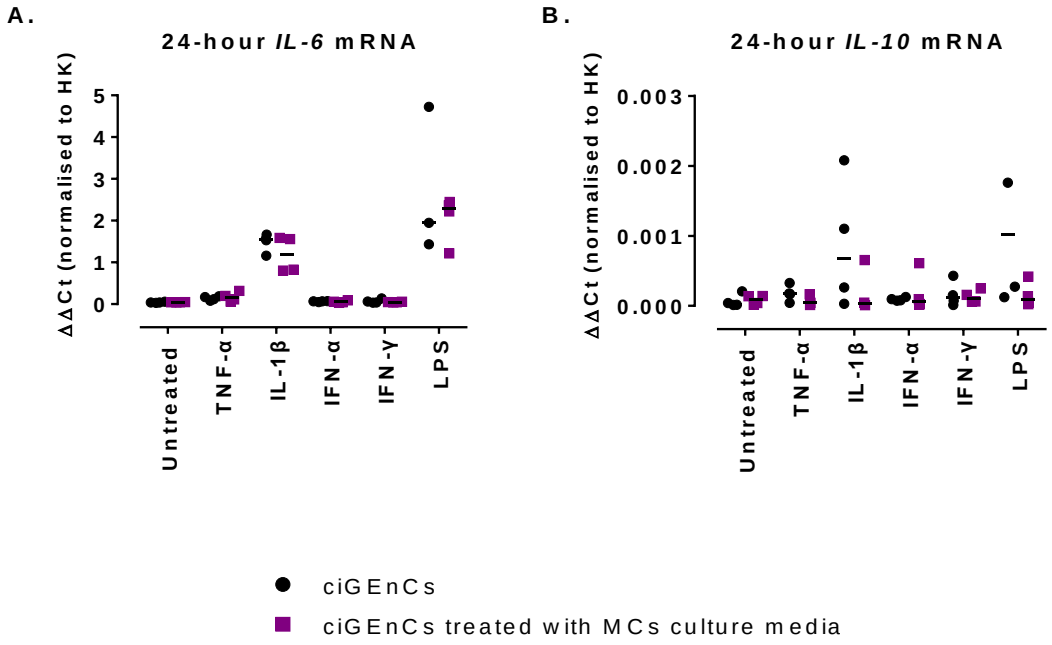


Figure 4.39 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated MC conditioned media on cytokine gene expression.

Changes in IL-6 (A), IL-10 (B) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs MCs conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

4.4.6.2.Chemokine mRNA expression after treatment with MCs conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS MCs conditioned media were not found to statistically significantly increase chemokine mRNA expression (**Figure 4.40**).

The untreated ciGEnCs' basal expression levels of *IL-8* (**Figure 4.40-A**), *MIP-1α* (**Figure 4.40-B**) and *IP-10* (**Figure 4.39-C**) were low and were not altered by the untreated MC media treatments. Similarly, no robust changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

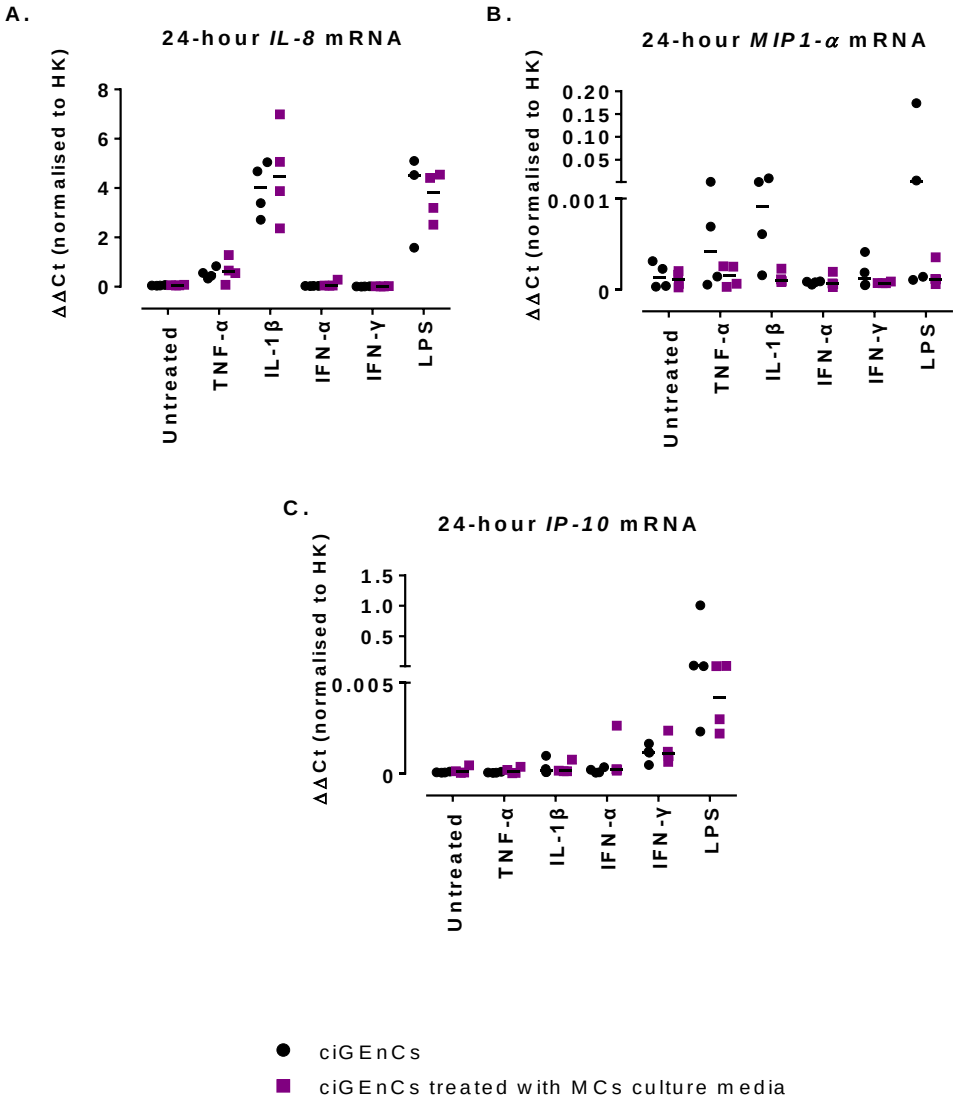


Figure 4.40 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated MC conditioned media on chemokine gene expression.

Changes in IL-8 (A), MIP-1α (B), IP-10 (C) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs MCs conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

4.4.6.3.Urinary biomarker mRNA expression after treatment with MCs conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS MCs conditioned media were not found to statistically significantly increase urinary biomarker mRNA expression (**Figure 4.41**).

The untreated ciGEnCs' basal expression levels of *MCP-1* (**Figure 4.41-A**) and *VCAM-1* (**Figure 4.41-B**) were low and were not altered by the untreated MC media treatments. Similarly, no robust changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

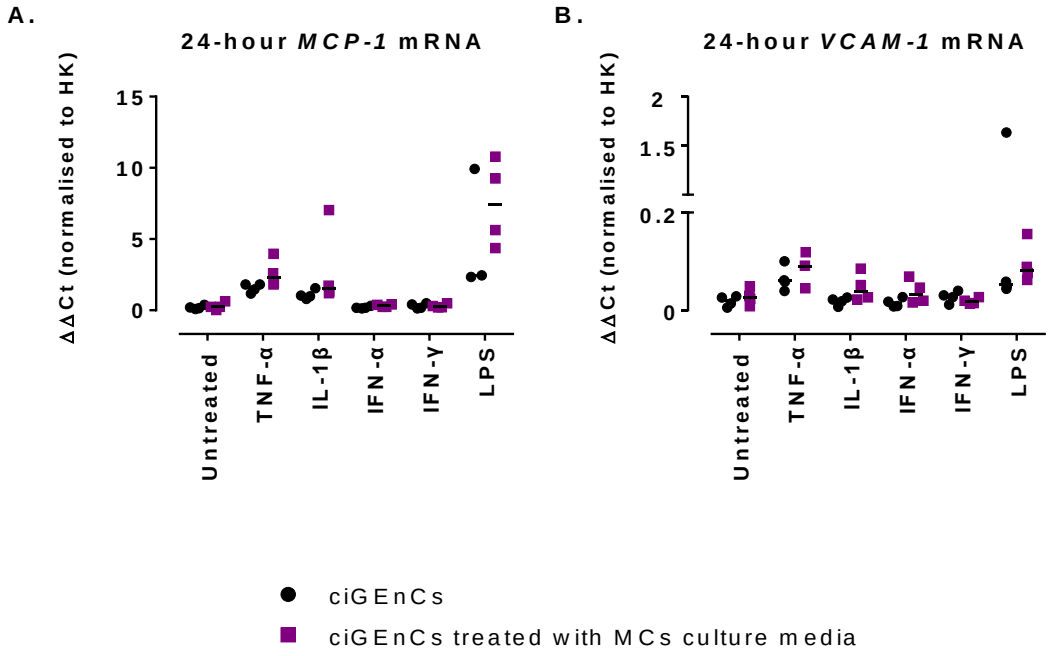


Figure 4.41 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated MC conditioned media on urinary biomarker gene expression.

Changes in MCP-1 (A), VCAM-1 (B) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs MCs conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. Fold changes in MCP-1 (C), VCAM-1 (D) mRNA expression. HK: YWHAZ.

4.4.6.4.Blood cell growth factor mRNA expression after treatment with MCs conditioned media

The untreated, TNF-α, IL-1β, IFN-α, IFN-γ and LPS MCs conditioned media were not found to statistically significantly increase blood cell growth factor mRNA expression (**Figure 4.42**).

The untreated ciGEnCs' basal expression levels of *M-CSF* (**Figure 4.42-A**) and *GM-CSF* (**Figure 4.42-B**) were low and were not altered by the untreated MC media treatments. Similarly, no robust changes were observed when cytokine/LPS ciGEnC treatments were compared to their cytokine/LPS-stimulated THP-1 conditioned media ciGEnC treatment counterparts.

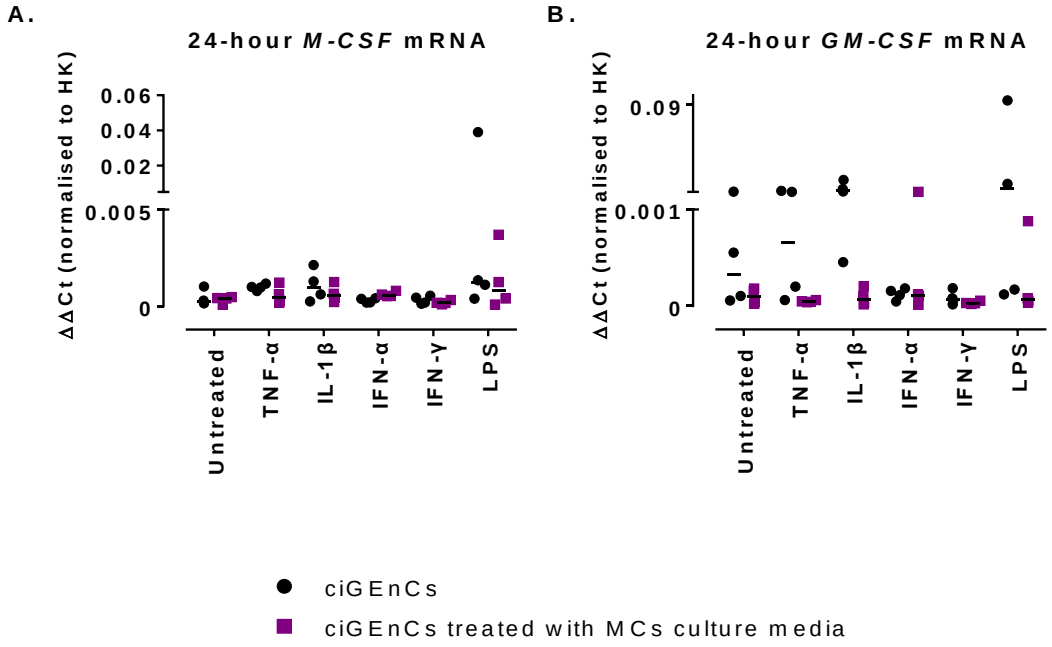


Figure 4.42 Effect of 24-hour ciGEnC treatment with untreated, TNF-α-, IL-1β-, IFN-α, IFN-γ- and LPS-treated MC conditioned media on blood cell growth factor gene expression.

Changes in M-CSF (A), GM-CSF (B) mRNA expression. Data presented as interleaved scatter plots are analysed by multiple t-tests with p-value corrections using the Holm-Sidak test (ciGEnCs vs MCs conditioned media-treated ciGEnCs: Untreated, TNF-α, IL-1β, IFN-α, IFN-γ, LPS). p=NS, N=4/group. HK: YWHAZ.

These findings could suggest that, at this (24h) time-point, the MCs are unable to contribute to the pro-inflammatory phenotype of the GEnCs potentially due to inability of adequate pro-inflammatory protein production.

4.5. Discussion

The experiments presented in this chapter, aimed to investigate how the renal microenvironment, including neighbouring glomerular cells, blood-circulating factors and infiltrating neutrophils and macrophages could affect the human GEnCs. Due to their pivotal role in pro-inflammatory protein production, as shown in Chapter 3, it was hypothesised that, through interacting with their surrounding environment, the human GEnCs would be able to sustain and reinforce their pro-inflammatory phenotype.

To verify this hypothesis in a physiologically relevant model, the ciGEnCs were treated for 4 and 24 hours with serum samples from JSLE patients with active and inactive LN and from healthy controls. This way, the potential effect on GEnCs exerted by the secreted factors released within the circulation (hormones, acute-phase proteins from the liver, dietary factors, secreted factors from other tissues and immune cell-derived pro-inflammatory factors) was tested. Furthermore, conditioned media from previously cytokine- or LPS-activated or untreated primary human neutrophils and macrophages, THP-1 macrophages, podocytes and MCs were used to stimulate the ciGEnCs for 24 hours. This way, the effect of secreted factors produced by cell types that can potentially interact with the GEnCs, was also investigated.

The 4-hour serum treatments did not reveal any significant changes in the expression levels of the genes tested. The 24-hour serum treatments did not induce significant increases in the mRNA expression levels of most pro-inflammatory genes tested. However, *IL-6* and to a lesser extent *IL-1β* mRNA levels were increased by the JSLE

sera compared to the HCs, whereas *IP-10* mRNA levels tended to be decreased by the JSLE sera. IL-6 and IL-1β are two important pro-inflammatory cytokines that could contribute to the sustainability of the renal pro-inflammatory microenvironment, as previously described in Chapter 1 (literature review findings) and Chapter 3 (data from the *in vitro* ciGEnC model developed for this thesis' experiments). It must be noted that the inactive and not the active LN serum treatments led to a decrease of pro-inflammatory gene expression (especially *TNF-α* and *IP-10*) compared to HCs. This may indicate a differential distribution of cytokines and chemokines between active and inactive LN sera. In addition, the 24-hour JSLE serum treatments were not able to modulate *L-PGDS* and *TF* mRNA expression, an outcome similar to that of cytokine or LPS treatments on these lowly expressed urinary biomarkers (Chapter 3). This finding corroborates the hypothesis that the human GEnCs are not the main source of all urinary biomarkers for LN but instead, they are specialising in producing the two biomarkers that are more closely related to pro-inflammatory responses; MCP-1 and VCAM-1.

The lack of more marked expression of cytokine responses induced by the JSLE sera could be because within the patient samples derived from patients taking part in the UK JSLE Cohort Study and Repository, most of the JSLE patients have well-managed disease, and/or are on various medications (**Table 4.1**) used for suppression of their disease, and therefore suppression of pro-inflammatory responses. Sample numbers precluded evaluation of any specific effect of medication on outcomes of experiments presented here.

Hydroxychloroquine (HCQ) is an anti-malarial drug that can inhibit T cell proliferation, production of pro-inflammatory cytokines such as IL-1, IL-6, IFN-α, IFN-γ and TNF-α and in SLE, its main mechanism of action is thought to be the inhibition of

Toll-like receptors, leading to inhibition of antigen-recognition[401]. Furthermore when combined with mycophenolate mofetil (MMF), HCQ has been shown to promote renal disease remission in SLE patients with LN[401,402]. MMF inhibits purine biosynthesis and the proliferation of activated lymphocytes and is involved in both the induction and maintenance of LN therapy[403].

In this study, 70% of the active LN patients and 70% of the inactive LN patients whose sera were used were administered with HCQ. Furthermore, 50% of active LN patients and 60% of inactive LN patients were administered with MMF. Both HCQ and MMF were administered at similar amounts in both active and inactive LN patients. It is therefore possible that lack of more robust results regarding *IL-6* and *IL-1β* induction after JSLE serum treatments and stratification of JSLE sera into active and inactive sera, could be attributed to the presence of HCQ and MMF within the sera.

Azathioprine is a purine analog that impedes immune cell proliferation and along with steroids, it is administered to LN patients[404]. Prednisolone is a corticosteroid medication that suppresses inflammatory responses via blocking leukocyte access and binding to the sites of inflammation and reducing the expression of adhesion molecules and the production of pro-inflammatory cytokines such as TNF-α and IL-6[405]. 30% of active LN patients and 20% of inactive LN patients whose sera were used to treat ciGEnCs in this study were administered with azathioprine at similar doses whereas 60% of active LN patients and 60% of inactive LN patients were also treated with prednisolone (again, at similar doses).

Rituximab is a chimeric anti-CD20 monoclonal Ab used to target mature B cells and pre-B cells, as B cell aberrant function leading to autoantibody production is one of the "hallmarks" of SLE[406]. Cyclophosphamide is a chemotherapy immunosuppressive

agent used for the treatment of patients with renal and central nervous system disease[407]. 10% of active LN patients but no inactive LN patients were administered with rituximab. Similarly, 10% of active LN patients but no inactive LN patients were administered with cyclophosphamide.

Finally, methotrexate is a folic acid antagonist that can impede immune cell proliferation[408] whereas intravenous immunoglobulin (IVIG) has an immunomodulatory effect and is known to block macrophage FcRs and inhibit immune cell functions such as T cell proliferation and dendritic cell differentiation[409]. In this study, 20% of inactive LN patients but no active LN patients received methotrexate whereas 10% of inactive LN patients but no active LN patients were administered with IVIG. As the majority of JSLE patients received a combination of medications at the time that the serum samples were taken, it is possible that a variety of pro-inflammatory responses had already been efficiently suppressed.

Similar to the pro-inflammatory genes tested, only a small number of the ECM-related genes tested were modified by the JSLE serum treatments when compared to the HC treatments. *LAMB2* expression by the ciGEnCs was significantly upregulated by the JSLE serum treatments and especially by the inactive LN serum treatments indicating that even during disease remission, pro-fibrotic changes might still be occurring. LAMB2 is one of the main components of the renal GBM, along with collagen type IV, heparan sulphate and nidogen[410,411]. Increased *LAMB2* expression could suggest potential alterations in the physiological proportions of the GBM components.

Furthermore, a trend was also observed for elevated ciGEnC *FN* mRNA levels after JSLE serum treatments compared to HC serum treatments. FN is the first ECM protein to be aberrantly accumulated and deposited in renal fibrosis[412] and physiologically it is

one of the main components of the MCs' ECM[410,413] but not of the main GBM components. Therefore, the elevated expression of *FN* mRNA by the ciGEnCs combined with the increased *LAMB2* expression implies a potential for the serum of JSLE patients to promote the initiation of pro-fibrotic events locally in the kidney via inducing excessive ECM protein deposition by the human GEnCs; failure of resolution of these events, could eventually lead to glomerulosclerosis. It had to be noted that a study using human GEnCs and HUVECs, indicated the capacity of FN to act as a "bridge" that facilitated the internalisation of IgG3 monoclonal antibodies (obtained by lupus-prone MRL/gld mice) by the human endothelial cells[414]. Therefore, the increased presence of FN within the renal microenvironment could promote LN-mediated glomerular injury via more than one possible way.

Finally, the levels of ciGEnC *COL1A1* mRNA expression were found to be significantly decreased by JSLE serum treatments compared to those of HCs. Decreased type I collagen production within the kidney during renal disease, could potentially lead to an increase in other type I collagen chains or other collagen types, such as collagen type III and IV. Therefore, although at 24 hours *COL3A1* and *COL4A1* mRNA levels were not significantly modified, more chronic exposure of ciGEnC to JSLE sera could increase their expression compared to HCs. Similarly, although *MMP* mRNA levels were not significantly modulated at the 24-hour time-point, more chronic exposure could spur increased MMP levels leading to remodeling of the basement membrane and glomerulosclerotic changes.

A limitation of this model was the low serum concentration used to induce responses. The 5% treatments were used to mimic the concentration of sera used in standard endothelial media. However, due to the limited sample volumes collected from

paediatric patients increased serum concentrations were unable to be assessed. Blood plasma, which consists of serum together with the fibrinogens, accounts for 55% of total human blood[1]. Therefore physiologically, inside the human body, the human serum is present in much higher amounts than the 5% human sera used for the *in vitro* treatment of ciGEnCs. This suggests that the concentration of pro-inflammatory factors in the serum samples used *in vitro* might have been too low to induce potent ciGEnC activation. Future work could test increased sera concentrations to determine if a greater effect could be demonstrated.

In a study using HUVECs as well as a terminally differentiated endothelial cell line (EA.hy926), uraemic serum samples from patients with CKD and normal (human autologous) serum samples were used to treat the endothelial cells for 24 hours[415]. The normal serum treatment was performed at a concentration of 2.5% whereas a concentration response was performed for the uraemic serum treatments, including 1%, 2.5%, 5% and 10%[415]. Apart from the 1% concentration that had no effect on the target protein tested in this study (Phospho-GSK-3β), the rest of the uraemic serum treatments (2.5%, 5% and 10%) had a positive concentration response effect on the target protein, whose production levels increased along with increasing serum concentrations[415]. In a similar *in vitro* study from the same group, 10% uraemic serum from CKD or 10% normal serum was used to treat HUVECs for 0, 6 and 12 hours, with the 6-hour time-point leading to statistically significant decrease of the target protein expression (stromal-cell derived factor-1, SDF-1) by the HUVECs[416].

Thus, 10% and 15% dose response as well as time response (especially including more chronic time-points) ciGEnC treatments, could be necessary to thoroughly investigate the *in vitro* effect of active and inactive LN serum on ciGEnCs. It would however be imprudent to come to definite conclusions, as in the above studies HUVECs

and not human GEnCs have been utilised and furthermore serum from human patients with other diseases than LN have been used for treatments.

With the JSLE serum treatments, the potential effect of pro-inflammatory and pro-fibrotic mediators including mediators secreted by immune cells was tested. However, investigation took place more specifically of the effect of human neutrophils and macrophages as these two innate immune cell types are among the first to reach the sites of inflammation from a very early stage, that of acute phase inflammation. Experimental GN murine models have clearly demonstrated that upon acute renal inflammation, neutrophil and monocyte/macrophage glomerular accumulation occurs[417–420]. Furthermore, with the use of mouse models, the importance of neutrophil-monocyte/macrophage interactions in the development of GN has been highlighted[418,421].

In humans, the use of healthy control renal biopsies has revealed the presence predominantly of monocytes/macrophages and secondarily of T cells within the healthy glomeruli[422,423]. Renal biopsies from patients with GN, have revealed that in crescentic and post-infectious GN the presence of monocytes and granulocytes was significantly increased within the glomeruli[422]. Furthermore, in a study where renal biopsies of SLE patients with GN (diffuse proliferative -active or sclerosing-, focal proliferative, mesangial, membranous and membranoproliferative GN) were studied, the presence of intra-capillary monocytes was significantly increased in diffuse proliferative GN[423]. In the same study, a strong correlation was found between infiltrating monocytes and the presence of subendothelial immune deposits, a finding that highlights the importance of the interactions between GEnCs and monocytes/macrophages[423].

In renal biopsies of patients with anti-neutrophil cytokine Ab (ANCA)-associated GN and myeloperoxidase (MPO)-ANCA-associated vasculitis (MPO-AAV), the levels of MPO deposition were examined[424]. Again, increased presence of leukocytes within the glomeruli were observed, with monocytes/macrophages being the predominant leukocyte population followed by neutrophils and T cells[424]. As MPO is a lysosomal enzyme predominantly expressed by neutrophils and also an important component of NETs, the majority of MPO was found to be located within the glomerular-infiltrating neutrophils and monocytes/macrophages[424]. However, considerable amounts of extracellular MPO was also detected in the glomeruli in areas of NETs existence and the glomerular endothelium was found to be MPO-positive, suggesting that potential MPO transfer from neutrophils and monocytes/macrophages or MPO internalisation by the GEnCs could contribute to glomerular renal injury[424]. It has therefore been made clear, by both experimental GN models and studies focused on human renal biopsies, that both neutrophils and macrophages are playing a crucial role in glomerular renal injury.

The role of neutrophils in LN, is further corroborated by data derived from a study using healthy control, rheumatoid arthritis (RA) and SLE sera to treat human neutrophils activated to produce NETs[425]. In this study, a subset of the SLE sera, derived from patients who had either developed LN or were prone to developing LN, strongly inhibited NET degradation due to either increased presence of DNAse inhibitor or increased presence of NET-binding antigens[425]. Apart from the prolonged exposure of NETs to the immune system due to defective clearance, human neutrophil-released enzymes, namely active human elastase and cathepsin G, have been shown to induce glomerular injury when *in vivo* infused in rats[426]. Finally it has to be noted that the importance of neutrophils in human LN is also pinpointed by the fact that plasma and urine NGAL levels, are used as a biomarker for global renal disease activity in JSLE

patients[427]. Of course, NGAL can also be produced by other immune cells and therefore this marker does not purely indicate neutrophil infiltration.

As the neutrophils act faster than the macrophages and they are the first immune cell type to accumulate to the inflamed tissues within a few minutes or a few hours[428], the whole blood isolated human neutrophils were stimulated for just one hour with TNF-α, IFN-γ and LPS. TNF-α, IFN-γ but also bacterial products are known to be important neutrophil priming agents (along with other cytokines or chemokines), leading to neutrophil activation[429,430]. Upon activation, human neutrophils are able to produce a variety of pro-inflammatory proteins, such as TNF-α, IL-1β, IL-6, IL-8, MCP-1, GM-CSF, IFN-α and IFN-γ[431]. These pro-inflammatory proteins could then recruit more immune cells -for example neutrophils or macrophages- to the sites of inflammation, or potentially influence cells found locally in the inflamed tissues, such as the human endothelium. In this study, the neutrophil conditioned media did not significantly increase any of the genes tested.

Classically activated M1-like macrophages[432] are accumulated within the kidneys early at the initiation of acute renal inflammation (up to 48 hours) in animal models of GN[420,433–435] and contribute to renal injury by producing pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6[436]. On the contrary, in more chronic stages of renal inflammation (later than the first 48 hours) the M1-like macrophages are replaced by M2-like macrophages that promote healing and contribute to renal fibrosis in cases where resolution of inflammation is not achieved[435]. IFN-γ and LPS are known to promote differentiation towards the M1 macrophage phenotype[437] and for this reason, both primary human macrophages and THP-1 macrophages were stimulated for 48 hours

with IFN-γ or LPS, as the 48-hour M1 polarisation time-point has been broadly used by other researchers.

In a study using human blood isolated macrophages, the macrophages were polarised into M1-like after a 48-hour of combined treatment of 20 ng/ml of IFN-γ and 100 ng/ml of LPS[438] whereas in another study using human macrophages, the latter were polarised into M1-like via a 48-hour incubation with 10 ng/ml of IFN-γ and 1 μg/ml of LPS[439]. Similarly, in a study using THP-1 cells, the cells were polarised after a 48-hour incubation with 20 ng/ml of IFN-γ and 100 ng/ml of LPS[440]. Although different concentrations of IFN-γ and LPS have been mentioned throughout the literature to be used for the primary and THP-1 macrophage M1 polarisation treatments, in the current study I carried out the polarisation treatments using 10 ng/ml IFN-γ and 1 μg/ml LPS concentrations that were previously used in Chapter 3 to activate the ciGEnCs. Furthermore, I did not use IFN-γ and LPS in combination but each one alone as both IFN-γ alone and LPS alone have been shown to be able to induce M1 macrophage polarisation[441–443]. Therefore, future studies could potentially focus on the combined effect or IFN-γ and LPS in human primary macrophage and THP-1 macrophage pro-inflammatory activation prior to usage of conditioned media to stimulate ciGEnCs.

With the aid of the primary human macrophage media model that facilitated the initial exploration of potential effects of macrophage conditioned media on ciGEnCs, the human THP-1 macrophage/ciGEnC *in vitro* model was developed. Conditioned media from untreated, IFN-γ- and LPS-treated THP-1 macrophages with or without the presence of anti-TNF-α Ab/IL-1Ra, were collected and were used to stimulate the ciGEnCs for 24 hours. The rationale behind this approach was to validate the effect of human macrophage-secreted TNF-α and IL-1β on the human GEnCs, as such an event

could possibly occur upon renal inflammation. The infiltrating macrophages could be activated via the local pro-inflammatory microenvironment to produce pro-inflammatory cytokines that could subsequently lead to GEnC activation.

With TNF-α and IL-1β being responsible for the upregulated production by the ciGEnCs of most pro-inflammatory proteins tested in Chapter 3, the *in vitro* THP-1 media/ciGEnCs model focused on investigating how the secretion of these two prototypical pro-inflammatory cytokines by the THP-1 cells would affect the ciGEnCs. After verifying that the THP-1 cells could secrete low, basal levels of TNF-α and IL-1β that were slightly elevated after IFN-γ stimulation and more robustly increased after LPS THP-1 stimulation, anti-TNF-α Ab and IL-1Ra were added onto the conditioned media to examine whether they would effectively block TNF-α and IL-1β action.

To ensure that the use of anti-human TNF-α Ab and human recombinant IL-1Ra would specifically target TNF-α and IL-1β respectively, ciGEnCs were initially stimulated for 30 minutes or 24 hours with either TNF-α, with or without anti-TNF-α Ab, or IL-1β, with or without IL-1Ra (***see Materials and Methods of this Chapter, 4.4.8***). The real-time PCR and ELISA assays performed, indicate that anti-TNF-α neutralising Ab and human recombinant IL-1Ra treatment abrogated the effect of TNF-α and IL-1β respectively, and markedly decreased the mRNA expression and protein secretion of pro-inflammatory genes and proteins previously shown to be upregulated by the two cytokines. In addition, TNF-α- and IL-1β-mediated NF-κB nuclear translocation was also inhibited after anti-TNF-α and IL-1Ra addition respectively, providing evidence that anti-TNF-α and anti-IL-1β treatments could efficiently inhibit the formation of a pro-inflammatory environment within the kidneys in LN.

The THP-1 conditioned media, were able to induce changes in the mRNA expression of pro-inflammatory genes that were generally more robust than those induced by the human neutrophil conditioned media and often represented not only remarkable fold-changes but statistically significant changes, too. This could be explained by the fact that neutrophil infiltration and action within the inflammatory sites precedes that of macrophages and usually is not as long lasting as that of the macrophages. Therefore, the neutrophils could pave the way via initiating GEnC activation and secreting factors that could attract infiltrating macrophages within the kidneys. As in this study the potential secretion of different pro-inflammatory or chemotactic factors by cytokine- and LPS-stimulated neutrophils has not been tested, future studies could further delve into neutrophil-produced pro-inflammatory mediators.

The expression of pro-inflammatory genes (*VCAM*-1, *TNFR2*) and the secretion of important pro-inflammatory proteins was found to be statistically significantly upregulated following THP-1 media activation; IL-6, IL-8, IP-10, MCP-1, sVCAM-1, M-CSF, GM-CSF. For some of the proteins (IP-10, M-CSF, and GM-CSF) upregulated by the THP-1 media, the addition of anti-TNF-α/IL-1Ra onto the media did not sufficiently reduce their expression or secretion.

This was also observed in a smaller extent with some of the pro-inflammatory genes tested and could be explained by the fact that other pro-inflammatory mediators present within the THP-1 media could predominantly affect the expression/production of certain target genes/proteins. Furthermore, IFN-γ or LPS used to stimulate the THP-1 cells, could have a direct and robust effect on the genes or proteins tested. Indeed, as shown in Chapter 3, LPS can increase M-CSF, GM-CSF and IP-10 secretion and IFN-γ can induce high IP-10 secretion levels. It has to be noted that, although IFN-γ did not alter the basal THP-1 TNF-α secretion levels and only induced moderate to low IL-1β secretion, the

addition of anti-TNF-α Ab/IL-1Ra onto the IFN-γ THP-1 media led in most cases to remarkable suppression of pro-inflammatory gene or protein suppression.

This could potentially be attributed to the fact that IFN-γ still present within the THP-1 media could induce TNF-α and IL-1β secretion by the ciGEnCs. ciGEnC - secreted TNF-α and IL-1β could act in an autocrine manner, further stimulating the ciGEnCs. As anti-TNF-α and IL-1Ra were added onto the THP-1 media in concentrations much higher (μg/ml) than those of THP-1 secreted TNF-α and IL-1β (pg/ml) to ensure efficient cytokine suppression, sufficient anti-TNF-α Ab/IL-1Ra quantities could be available to also block the ciGEnC-produced TNF-α and IL-1β. Furthermore, the effect of the basal TNF-α and IL-1-β THP-1 secreted levels on ciGEnCs should not be underestimated as the untreated THP-1 media were also found to increase the expression of a number of pro-inflammatory molecules by the human ciGEnCs.

Using the THP-1 media/ciGEnC *in vitro* model, helped identify TNF-α and IL-1β macrophage secretion as an important event towards GEnC activation and demonstrate the effectiveness of anti-TNF-α Ab/IL-1Ra treatments *in vitro*, in terms of ciGEnC and macrophage targeting as a potential future therapeutic approach. As this model was the one leading to the most robust ciGEnC activation, a simplified schematic representation is illustrated on **Figure 4.43**. Future studies should focus on elucidating how individual addition of anti-TNF-α Ab and individual addition of IL-1Ra onto the THP-1 media could affect the production of pro-inflammatory genes and proteins. This way, the exact contribution of THP-1-secreted TNF-α and IL-1β on ciGEnC activation could be determined.

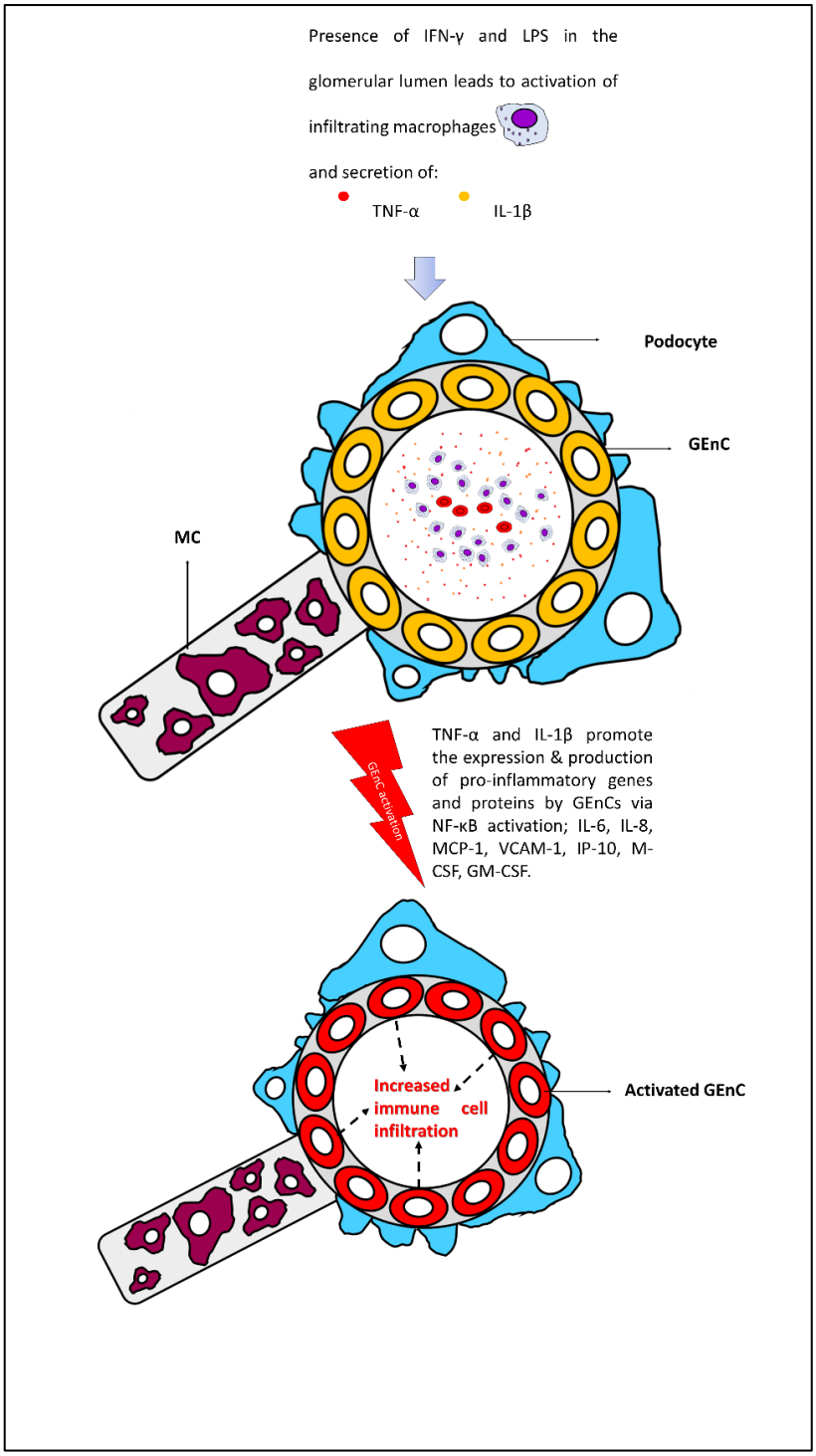


Figure 4.43 Potential effect of TNF-α and IL-1β secreting macrophages to GEnCs based on in vitro findings.

Furthermore, in this experimental setup the concentration of THP-1-secreted TNF-α and IL-1β was much lower (pg/ml) than that used for ciGEnC *in vitro* stimulation in Chapter 3 (10 ng/ml). Therefore, the full extent of the THP-1 activating effect on

ciGEnCs might have not been elucidated, due to experimental limitations. It is, however, very important to take into consideration that as also shown with the TNF-α and IL-1β concentration response qRT-PCR assays in Chapter 3, the ciGEnCs are satisfactorily responsive and can get activated even by low cytokine concentrations. Of course, more pro-inflammatory factors than just TNF-α and IL-1β present within the THP-1 media could affect the ciGEnCs and future work should also focus on identifying more macrophage-derived ciGEnC pro-inflammatory activators.

It is also important to mention that as the media containing the highest TNF-α and IL-1β THP-1-secreted levels were the LPS THP-1 media, their ability to activate NF-κB was tested via IF. As a result, the ciGEnCs treated with LPS THP-1 media were found to promote NF-κB nuclear translocation more robustly compared to ciGEnCs treated with LPS alone or ciGEnCs treated with LPS THP-1 media with anti-TNF-α/IL-1Ra addition. Therefore, the *in vitro* data provide evidence for macrophage-secreted TNF-α- and IL-1β-induced GEnC pro-inflammatory activation, via NF-κB activation and nuclear translocation.

The chimeric TNF-α blocking Ab infliximab, has been broadly used to treat human SLE and LN, although adverse effects have been reported[444] and have been presented at this thesis' Introduction. Therefore, *in vitro* findings in favour of anti-TNF-α treatments should always be treated with caution as an *in vitro* model cannot realistically imitate the complexity of the cellular and molecular structures within the human body. Anakinra (Kineret), a recombinant IL1-Ra, is widely used as a first-line treatment in juvenile-onset systemic idiopathic arthritis (JIA) with reportedly high efficacy and safety rates [445,446]. However, Anakinra is not currently used in JSLE patient treatment and further validation using more complex *in vitro* and *in vivo* models and clinical evidence is necessary to decide whether IL-1Ra could be used for JSLE treatment.

The podocyte and MCs conditioned media had the least robust effect on *in vitro* ciGEnC activation and did not induce any statistically significant changes in pro-inflammatory gene expression. It is possible that the main role of podocytes or MCs in renal inflammation is not that of the main pro-inflammatory contributors, or, that the production of pro-inflammatory factors by the podocytes and MCs is more limited compared to human GEnCs. In fact, research from our laboratory has shown that under pro-inflammatory stimuli human conditionally immortalised podocytes can secrete certain pro-inflammatory proteins[447].

As it has already been shown in this thesis, the human GEnCs are important intra-renal pro-inflammatory players. Thus, among the renal glomerular cells in LN, the GEnCs could play a major role in pro-inflammatory protein production whereas the podocytes and MCs could dominate other routes of renal pathogenesis and destruction, such as; GFB destruction and proteinuria for the podocytes[448] and fibrosis and renal scarring for the MCs[449]. Thus, future studies using pro-inflammatory cytokine-producing ciGEnC conditioned media for podocyte and MC activation, could generate very interesting results. Furthermore, it is important to note that although no paracrine podocyte and MC effect was observed on ciGEnCs, the cell-cell interactions among the three renal cell types should be investigated in the future as interesting findings could potentially arise.

Taken together, the data generated in this chapter, indicate that the human GEnCs can be affected by their surrounding microenvironment. Under inflammatory conditions such as in LN, infiltrating immune cells and neighbouring renal cells could actively promote GEnC activation. The activated GEnCs, in turn, will produce higher levels of pro-inflammatory proteins that will recruit more immune cells within the glomeruli but in the same time will enhance podocyte and MC activation. A positive pro-inflammatory

feedback loop could therefore be created within the kidneys, with the human GEnCs acting as the "bridge" between the circulation and the renal tissue.

4.6. Conclusions

In conclusion, the human ciGEnCs did not exhibit a strong pro-inflammatory or pro-fibrotic phenotype following treatments with JSLE patient sera, although *IL-6*, *IL-1β*, *FN* and *LAMB2* mRNA expressions were increased. Similarly, ciGEnC treatments with human neutrophil, podocyte and MCs conditioned media did not induce a pro-inflammatory response by the ciGEnCs. The THP-1 media, however, were able to promote a ciGEnC pro-inflammatory response, highlighting the importance of macrophages in renal disease.

5. Chapter 5: Investigating the effect of urinary biomarkers on human ciGEnCs

5.1. Introduction

The human ciGEnCs are potent *in vitro* producers of only two (MCP-1 and sVCAM-1) of the seven potential urinary biomarkers for active juvenile-onset LN investigated here, as identified previously in our laboratory[326,327]. The ciGEnCs also express in very low levels both TF and L-PGDS which, as shown in Chapters 3 and 4, were not affected or modified by either cytokine and LPS or serum treatments. Thus, the human GEnCs are not the main source of these urinary biomarkers within the kidney. It is most likely that their origin is either from other types of resident renal cells (although human podocytes have also been shown by our laboratory to not be the main source of the biomarkers) or from infiltrating and/or circulating immune cells[447]. Alternatively, the increase in the level of the biomarkers could be systemically induced and due to previous renal damage, the biomarkers could then be passively filtered through the glomerulus.

A plausible scenario could be that kidney infiltrating immune cell populations such as macrophages, could be activated within the nephritic kidney and subsequently release these biomarkers. With the human GEnCs being on the front line of the glomerulus' defence against blood circulating pro-inflammatory factors, the human GEnCs could eventually be affected by the presence of increasing numbers of urinary biomarkers in the glomerular lumen. Subsequently, biomarker-mediated alterations in the activation status of human GEnCs could contribute to GFB destruction, allowing for the passage of the biomarkers to the urinary space and, eventually, to their increased presence within the urine of LN patients.

Exploring the effect of the LN urinary biomarkers on human GEnCs could lead to findings explaining how intra-glomerular changes during LN renal flares contribute to the increased presence of these biomarker in the urine of JSLE patients.

5.2. Aim

To investigate the potential effect of the novel urinary biomarkers for LN on GEnC pro-inflammatory profile using an *in vitro* ciGEnC model.

5.2.1. Objectives

Objective 1: To assess whether treatments with human recombinant urinary biomarkers could induce a pro-inflammatory phenotype on ciGEnCs.

To address this objective, the ciGEnCs were *in vitro* stimulated with human recombinant biomarkers, individually or in combination, and the expression and/or production of pro-inflammatory genes/proteins was tested.

O**bjective 2:** To determine whether the human recombinant urinary biomarker treatments can affect the ciGEnC viability.

To address this objective, the ciGEnCs were *in vitro* stimulated with the human recombinant biomarkers in combination, and their apoptosis/necrosis levels were tested.

5.2.2. Chapter Hypothesis

The urinary biomarkers for LN lead to pro-inflammatory activation of human GEnCs, promoting a sustained inflammatory renal microenvironment.

5.3. Materials and Methods

5.3.1. ciGEnC preparation and treatment with urinary biomarkers

Fully differentiated ciGEnCs were seeded in 12-well plates for all treatments. Subsequently, they were incubated at 37^{o}C (with 5% CO_2) for 24 hours with human recombinant urinary biomarkers (AGP, CP, TF, L-PGDS, NGAL and MCP-1), alone or in combination. A minimum of three consecutive passages of ciGEnCs was used for all experiments.

5.3.1.1.Human recombinant biomarker treatments

The ciGEnCs were treated for 24 hours with six of the seven human recombinant urinary biomarkers for active LN; AGP, CP, TF, L-PGDS, MCP-1 and NGAL. VCAM-1 was not used to treat the ciGEnCs, as the human ciGEnCs were found to be potent *in vitro* producers of both sVCAM-1 and surface VCAM-1 and a literature search did not reveal research findings for a potential effect of VCAM-1 on human endothelial cells. Therefore, the human GEnCs could rather affect their surrounding environment via producing VCAM-1 and not the opposite. Although MCP-1 is also potently secreted by stimulated human ciGEnCs *in vitro*, it was still used to treat the ciGEnCs, as MCP-1 has been shown to induce HUVEC apoptosis *in vitro* [450]. The concentrations used for biomarker treatments reflected those found present in the urine of patients with active LN (high) and those found present in the urine of patients with inactive LN (low)[327,451] and are presented in **Table 5.1**.

Urinary Biomarker	High concentration *Urine of JSLE patients with active LN*	Low concentration *Urine of JSLE patients with inactive LN*
Alpha-1-acid-glycoprotein (AGP)	20,600 ng/ml	300 ng/ml
Ceruloplasmin (CP)	4,700 ng/ml	700 ng/ml
Transferrin (TF)	4,900 ng/ml	1,200 ng/ml
Lipocalin-Type Prostaglandin D Synthase (L-PGDS)	1,200 ng/ml	300 ng/ml
Monocyte Chemoattractant Protein-1 (MCP-1)	0.26 ng/ml	0.172 ng/ml
Neutrophil gelatinase-associated lipocalin (NGAL)	34 ng/ml	22.2 ng/ml

Table 5.1 Concentrations of urinary biomarkers used in in vitro experiments.

5.3.2. qRT-PCR analysis to test changes in pro-inflammatory gene expression in biomarker-treated ciGEnCs

RNA from the biomarker-treated ciGEnCs was extracted and transcribed to cDNA as previously described in the **Materials and Methods** (**section 2.3**). This cDNA was used for qRT-PCR analysis to investigate changes in mRNA expression levels for genes listed in **Table 5.2.**

Gene	Forward Primer Sequence (5'- 3')	Reverse Primer Sequence (5'- 3')
ACTB	CATTGCGGTGGACGATGGA	AGATCAAGATCATTGCTCCTCCTG
TBP	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
TUBB	GGACCGCATCTCTGTGTACT	CTGCCCCAGACTGACCAAATA
TNF-α	CTTCTGCCTGCTGCACTTTG	GGGTTTGCTACAACATGGGC
IL-6	AGAGGCACTGGCAGAAAACA	TCACCAGGCAAGTCTCCTCA
IL-8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA
MIP-1α	CATTCCGTCACCTGCTCAGA	AGCAGCAAGTGATGCAGAGA
IP-10	AGCCCCACGTTTTCTGAGAC	GAGAGAGGTACTCCTTGAATGCC
M-CSF	CCCCAGTCCTCTCTTAAAAGGC	GAGAGGACCCAGGCAAACTT
GM-CSF	GAGACACTGCTGCTGAGATGAA	AGGAAGTTTCCGGGGGTTGGAG
MCP-1	CTCGCTCAGCCAGATGCAAT	TCTCCTTGGCCACAATGGTC
VCAM-1	GGTGGGACACAAATAAGGGT	GCTTGAGAAGCTGCAAAC
TNFR1	CTGGAGCTGTTGGTGGGAAT	TTGTGGCACTTGGTACAGCA
TNFR2	CACCGGGAGCTCAGATTCTTC	CACTGTGAGCTGTGGTCAGA
IL-1RI	GCGGCAGGAATGTGACAA	TACCAAGGGGTCCAGCTTCT
IL-1RII	TGAGCAGCAACAAGGCCA	CAACACGTACAAGCGCAACA

Table 5.2 Primers used for detection of urinary biomarker expression by human macrophages; ACTB, TBP and TUBB were used as HK genes.

5.3.3. ELISA assays to test changes in pro-inflammatory protein secretion in biomarker-treated ciGEnCs

The secretion of MCP-1, sVCAM-1, IL-6, IL-8, IL-10, M-CSF, GM-CSF, MIP-1α, IP-10, TNF-α and IFN-γ was tested with single ELISAs or a Luminex multiplex ELISA assay, utilising conditioned media from ciGEnCs treated for 24 hours with the six human recombinant biomarkers at high and low concentrations.

5.3.4. Primary human macrophage culture and potential biomarker mRNA expression

PBMCs were isolated from whole-blood samples of healthy volunteers as previously described in the **Materials & Methods** and were re-suspended in RPMI (+10% FBS). They were then seeded into 12-well plates at 37°C for 2 hours. Following this, the non-adherent lymphocytes were removed, and the adherent monocytes were treated with RPMI (+10% FBS, 1% penicillin-streptomycin and 50ng/mL M-CSF) to aid their differentiation into macrophages. These were then incubated at 37°C for 7 days. Macrophage differentiation was confirmed using light microscopy, as presented in **Figure 4.1** of **Chapter 4**.

Macrophages (MΦ) were then treated for 48h with 10ng/mL IFN-γ or 1μg/mL LPS at 37°C. Following this, the conditioned media were removed, and RNA was extracted and transcribed to cDNA as previously described in **section 2.3**. This cDNA was used for qRT-PCR analysis to investigate MΦ-derived urinary biomarker mRNA expression. The primers used are listed on **Table 5.3.**

Gene	Forward Primer Sequence (5'- 3')	Reverse Primer Sequence (5'- 3')
TUBB	GGACCGCATCTCTGTGTACT	CTGCCCCAGACTGACCAAATA
AGP	GGCCTTTCGAAACGAGGAGT	CGCTGGACATTCAGGTAGGT
CP	ACAGCACCTGGAAGTGACTC	AGGGCCTCTCTCCTTTCGAT
TF	TTCACTGGCCCAAGTGGTTT	AGAAGGAAGGGCTCAGTTGC
L-PGDS	CCAGGGCTGAGTTAAAGGAGA	CCCTGGGGAGTCCTATTGTTC
NGAL	ACCCTCTACGGGAGAACCAA	AGCTCCCTCAATGGTGTTCG
MCP-1	CTCGCTCAGCCAGATGCAAT	TCTCCTTGGCCACAATGGTC
VCAM-1	GGTGGGACACAAATAAGGGT	GCTTGAGAAGCTGCAAAC

Table 5.3 Primers used for detection of urinary biomarker expression by human macrophages; TUBB was used as a house keeping gene.

5.3.5. ciGEnC viability following biomarker treatments

The ciGEnCs were treated for 24 hours with all human recombinant biomarkers, combined at either high or low concentrations. Subsequently, the ciGEnCs were trypsinised, centrifuged at 300 xg for 5 minutes and the cell pellets were resuspended in HBSS and initially stained with Annexin V – FITC (15 minutes on ice) and then with PI (5 minutes, room temperature, dark) to perform a flow cytometric apoptosis assay.

5.3.6. Statistical analyses

Changes among three (or more) groups were studied using Kruskal-Wallis test with Dunn's post-hoc test. In all cases a P value ≤ 0.05 was considered significant to reject the null hypothesis and differences were considered significant.

5.4. Results

5.4.1. Human macrophages can express increased levels of mRNA of urinary biomarkers after IFN-γ or LPS stimulation

To identify a potent cellular source of the LN urinary biomarkers, PBMC-isolated primary human macrophages (MΦ) were treated for 48 hours with either IFN-γ or LPS (untreated macrophages were always included). Subsequently, changes in the mRNA expression of *AGP*, *CP*, *TF*, *VCAM-1*, *MCP-1* and *NGAL* were tested.

The primary human macrophages were found not to express mRNA for *NGAL*. However, they did express mRNA for the other human urinary biomarkers tested. More specifically, the LPS treatments significantly increased *AGP* MΦ mRNA (0.001 [0-0.01], p=0.01) compared to untreated MΦ (0 [0 - $4x10^{-6}$]) (**Figure 5.1-A**). *CP* mRNA expression was also statistically increased by LPS treatments (0.007 [0.0008-0.02], p=0.005) compared to untreated MΦ (0.0001 [0-0.0003]) (**Figure 5.1-B**). The human macrophages were also able to express *TF* and *L-PGDS* but the IFN-γ and LPS treatments did not statistically significantly alter the basal expression levels of *TF* and *L-PGDS*. (**Figure 5.1-C & D**). Finally, the IFN-γ-treated MΦ expressed statistically significantly higher levels of *VCAM-1* (0.3 [0.007-0.1], p=0.008) (**Figure 5.1-E**) and *MCP-1* mRNA (1.3 [0.7-2.3], p=0.03]) (**Figure 5.1-F**) compared to the untreated MΦ (*VCAM-1*: 0.0002 [$1x10^{-5}$-0.001], *MCP-1*: 0.3 [0.04-0.8]).

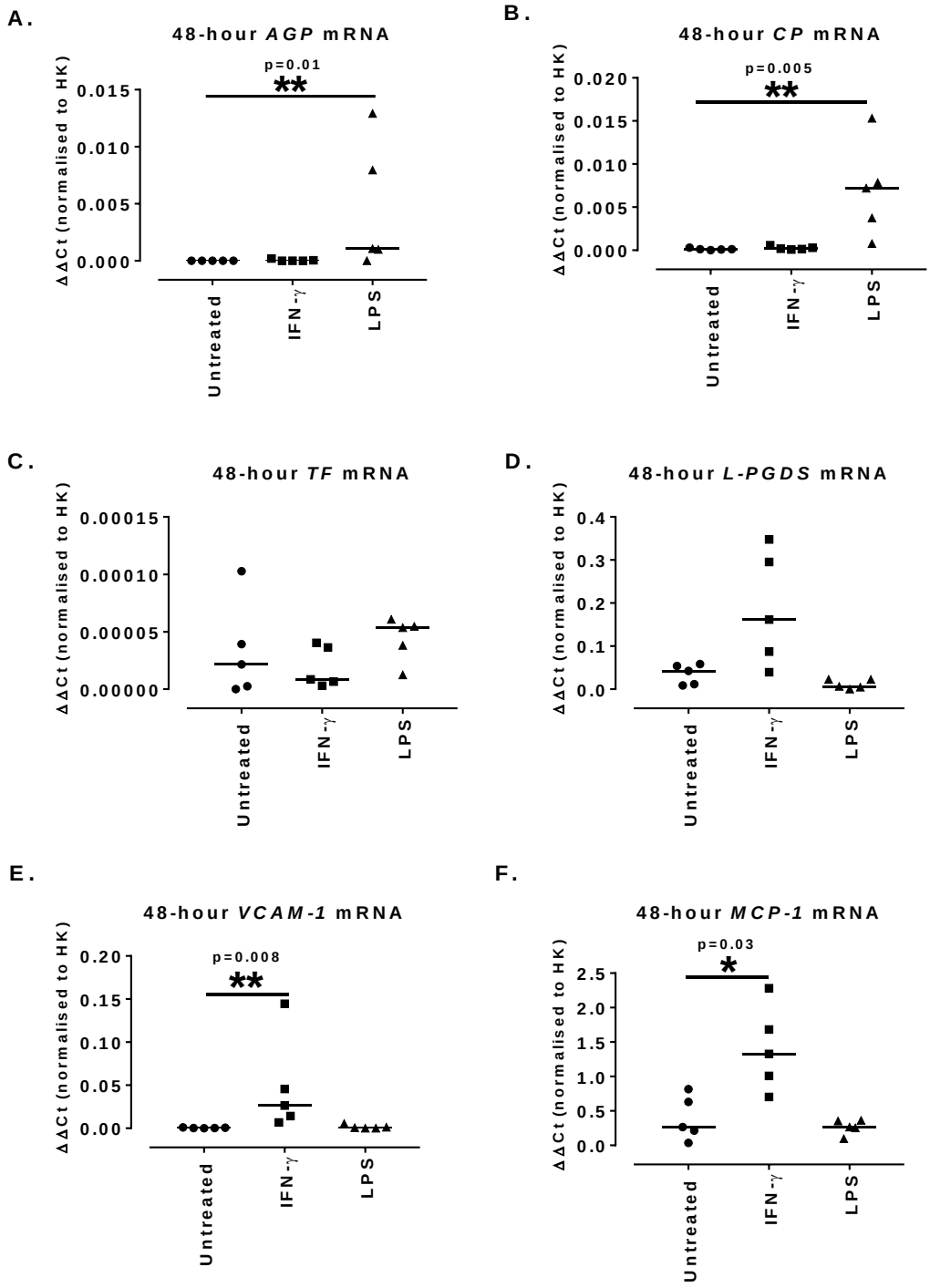


Figure 5.1 qRT-PCR for MCP-1, VCAM-1, L-PGDS and TF mRNA after 48-hour treatments with cytokines and LPS.

*A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in L-PGDS mRNA levels. D. Changes in TF mRNA levels N=5-6/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05, **p≤0.01 vs untreated. HK: TBP, TUBB.*

5.4.2. Individual biomarker treatments did not induce any changes in the mRNA expression of pro-inflammatory genes

Before assessing the effect of combined biomarker treatment on the expression of pro-inflammatory genes by the ciGEnCs, the effect of each biomarker alone (AGP, CP, L-PGDS, TF, MCP-1, NGAL) after 24-hour incubations was also tested. No substantial changes, however, were found in the mRNA expression of the pro-inflammatory genes tested (N.B. the same genes tested on Chapter 3 following TNF-α, IL-1β, IL-13 and IFN-γ ciGEnC stimulation and concentration response treatments) after either high or low biomarker concentrations were used.

5.4.2.1.The effect of AGP on the pro-inflammatory gene expression

The baseline mRNA levels of pro-inflammatory cytokines and chemokines expressed by the untreated ciGEnCs were not altered either by AGP high (20,600ng/mL) or AGP low (300ng/mL) 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of AGP high and low concentration treatments on *IL-6* (**Figure 5.2-A**), *IL-8* (**Figure 5.2-B**), *MIP-1α* (**Figure 5.2-C**) and *IP-10* mRNA levels (**Figure 5.2-D**), were compared to basal expression levels in the untreated ciGEnCs.

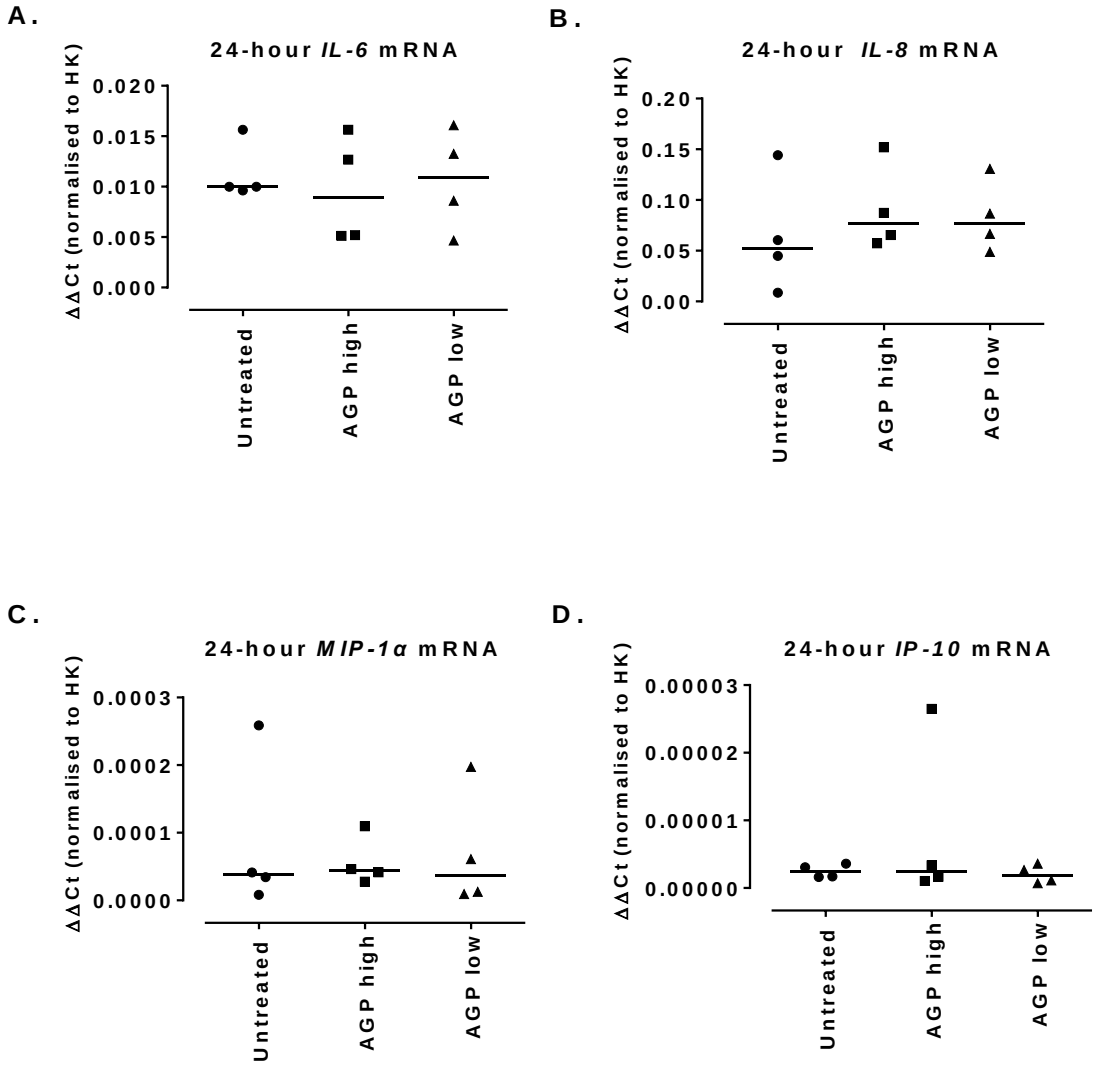


Figure 5.2 qRT-PCR for pro-inflammatory cytokine and chemokine expression after 24-hour treatments with AGP high and low concentrations.

A. Changes in IL-6 mRNA levels. B. Changes in IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes in IP-10 mRNA levels N=4/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS vs untreated. HK: ACTB, TBP, TUBB.

The baseline mRNA levels of urinary biomarkers and blood cell growth factors expressed by the untreated ciGEnCs were not altered either by AGP high or AGP low 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of AGP high and low concentration treatments on *MCP-1* (**Figure 5.3-**

A), *VCAM-1* (**Figure 5.3-B**), *M-CSF* (**Figure 5.3-C**) and *GM-CSF* (**Figure 5.3-D**) were compared to the basal expression levels in untreated ciGEnCs.

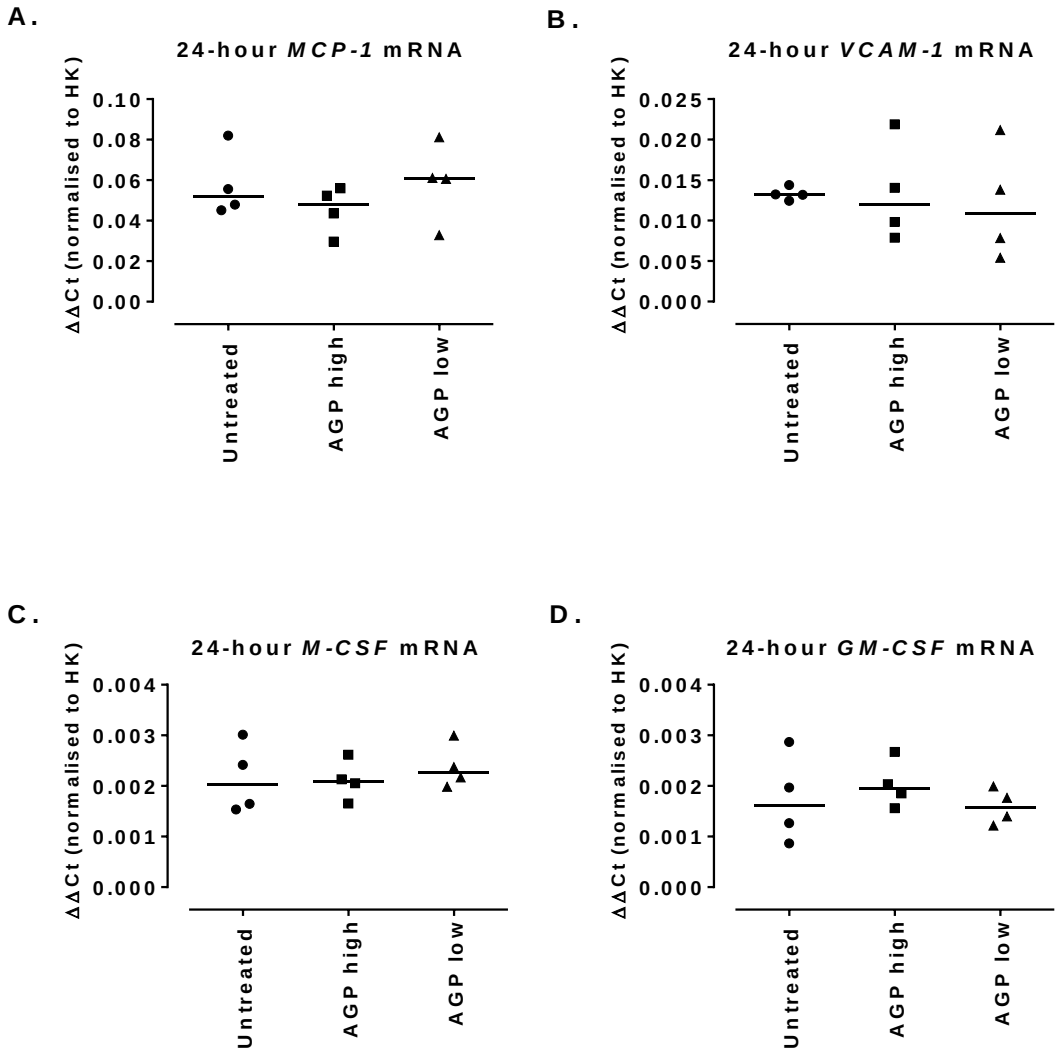


Figure 5.3 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with AGP high and low concentrations.

A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=4/group. Data presented as median ΔΔCt are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.

5.4.2.2.The effect of CP on the pro-inflammatory gene expression.

The basal levels of pro-inflammatory cytokine and chemokine mRNA expression were not altered either by CP high (4,700 ng/ml) or CP low (700 ng/ml) 24-hour treatments. More specifically, no statistically significant changes were observed for *IL-6* (**Figure 5.4-A**), *IL-8* (**Figure 5.4-B**), *MIP-1α* (**Figure 5.4-C**) and *IP-10* mRNA levels (**Figure 5.4-D**) after CP treatments when compared to the expression levels in untreated ciGEnCs.

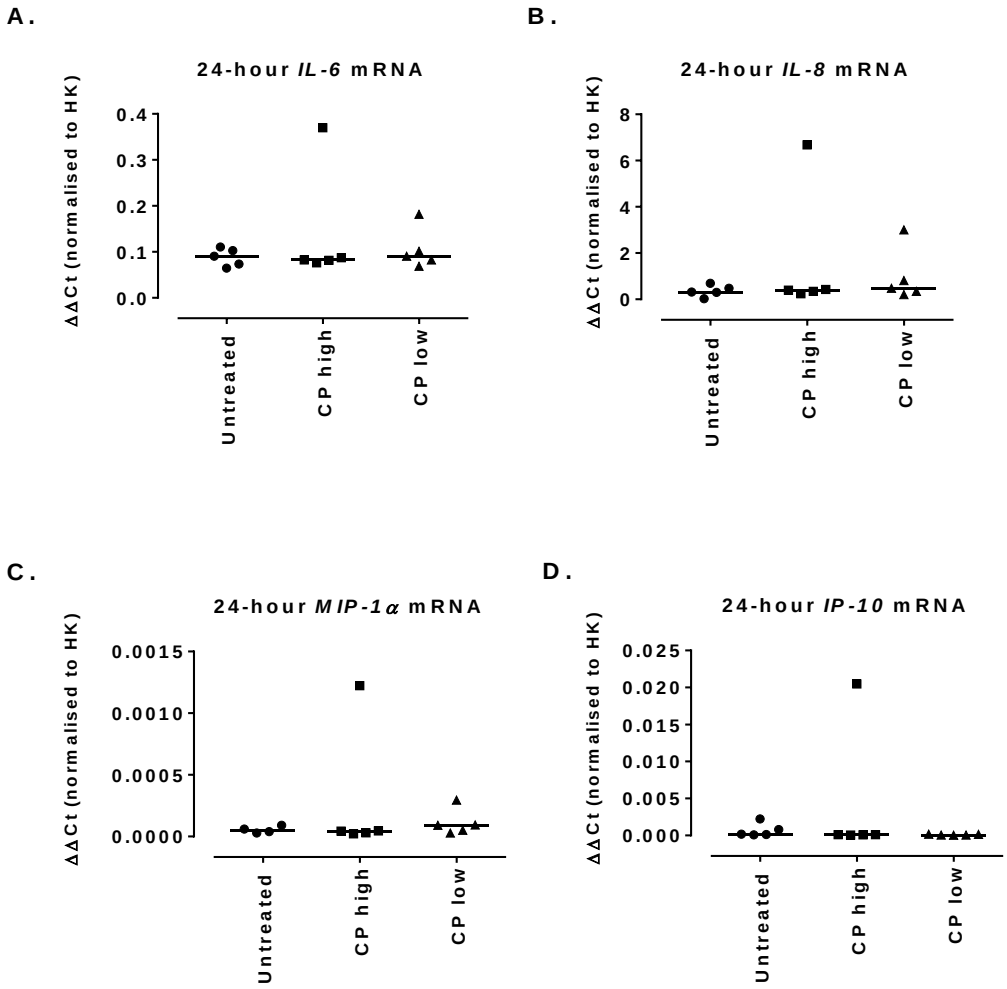


Figure 5.4 qRT-PCR for cytokine and chemokine expression after 24-hour treatments with CP high and low concentrations.

A. Changes in IL-6 mRNA levels. B. Changes IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes IP-10 mRNA levels. N=5/group. Data presented as median ΔΔCt are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS vs untreated. HK: ACTB, TBP, TUBB.

The basal levels of pro-inflammatory urinary biomarker and blood cell growth factor mRNA expression were not altered either by CP high or CP low 24-hour treatments. More specifically, no statistically significant changes were observed for *MCP-1* (**Figure 5.5-A**) or *VCAM-1* (**Figure 5.5-B**) after CP treatments when those were compared to the untreated ciGEnCs. Similarly, the CP high and low concentrations were not able to modulate the basal mRNA expression levels of *M-CSF* (**Figure 5.5-C**) and *GM-CSF* (**Figure 5.5-C**) in ciGEnCs.

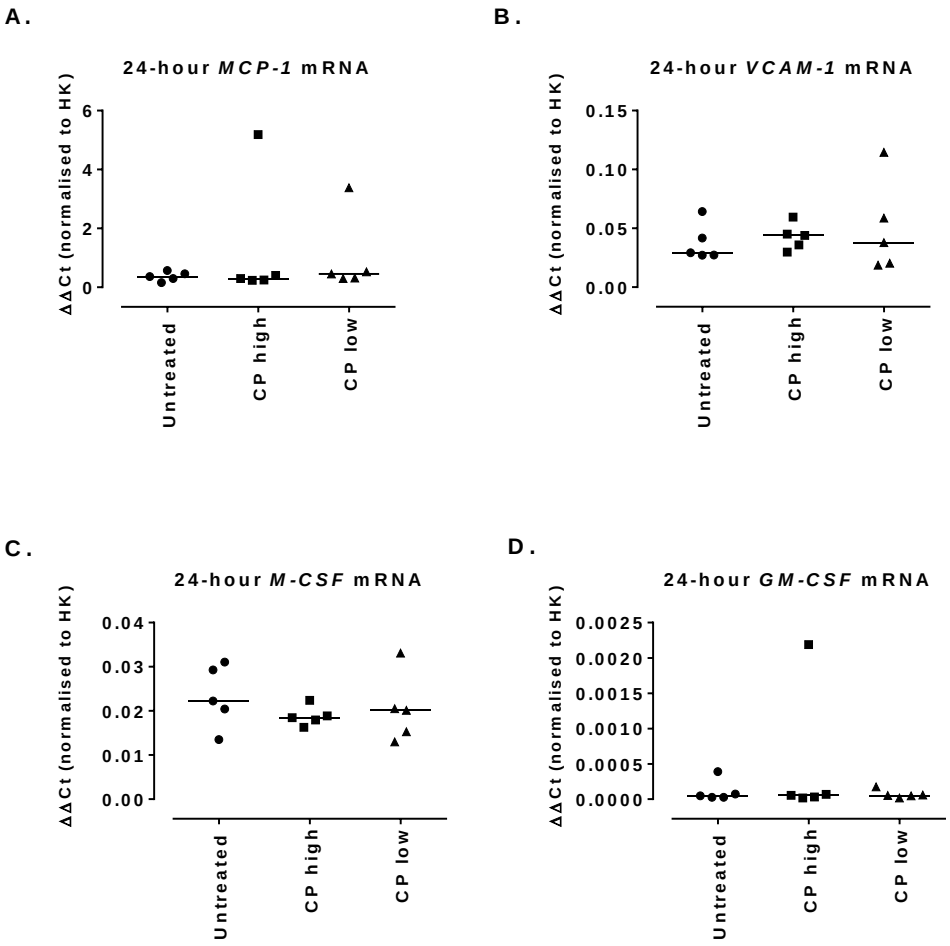


Figure 5.5 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with CP high and low concentrations.

A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=4/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.

5.4.2.3.The effect of TF on the pro-inflammatory gene expression

The basal mRNA expression levels of cytokines and chemokines (expressed by the untreated ciGEnCs) were not modified either by TF high (4,900 ng/ml) or TF low (1,200 ng/ml) 24-hour treatments. More specifically, no statistically significant changes in mRNA expression were observed when the effects of TF high and low concentration treatments on *IL-6* (**Figure 5.6-A**), *IL-8* (**Figure 5.6-B**), *MIP-1α* (**Figure 5.6-C**) *and IP-10* (**Figure 5.6-D**) were compared to the untreated ciGEnCs.

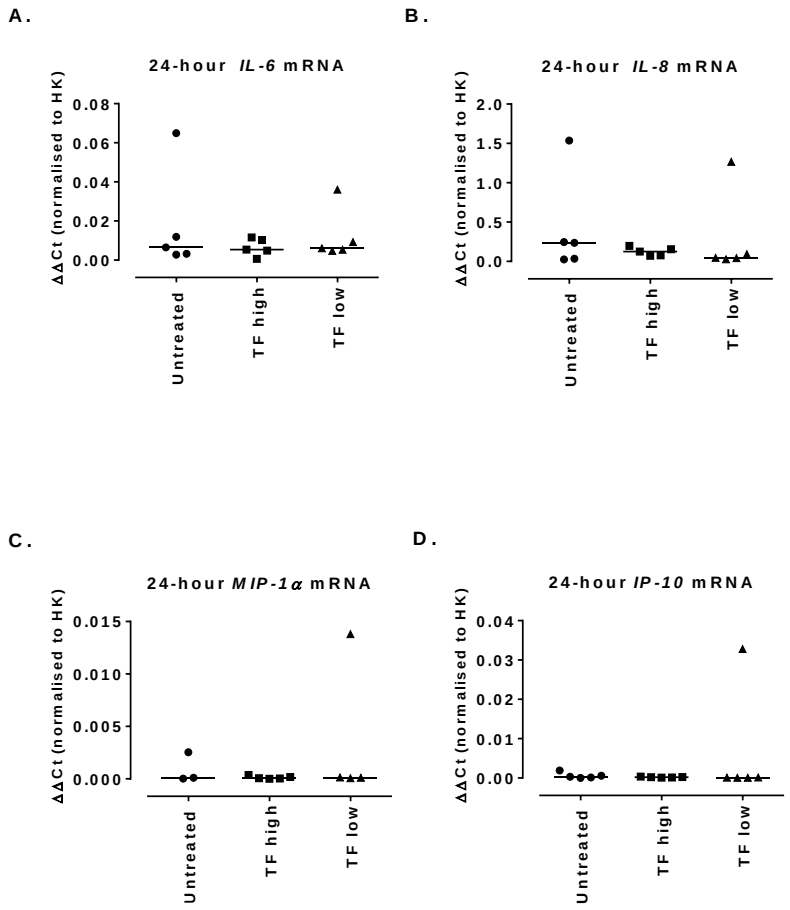


Figure 5.6 qRT-PCR for cytokine and chemokine expression after 24-hour treatments with TF high and low concentrations.

A. Changes in IL-6 mRNA levels. B. Changes IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes IP-10 mRNA levels. N=5/group. Data presented as median ΔΔC, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS vs untreated. HK: ACTB, TBP, TUBB.

The mRNA levels of urinary biomarkers expressed by the untreated ciGEnCs were not affected either by TF high or TF low 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of TF high and TF low concentration treatments on *MCP-1* (**Figure 5.7-A**) and *VCAM-1* (**Figure 5.7-B**) were compared to the untreated ciGEnCs. Similarly, the TF high and low concentrations did not significantly change the expression of the blood cell growth factors, *M-CSF* (**Figure 5.7-C**) and *GM-CSF* (**Figure 5.7-D**).

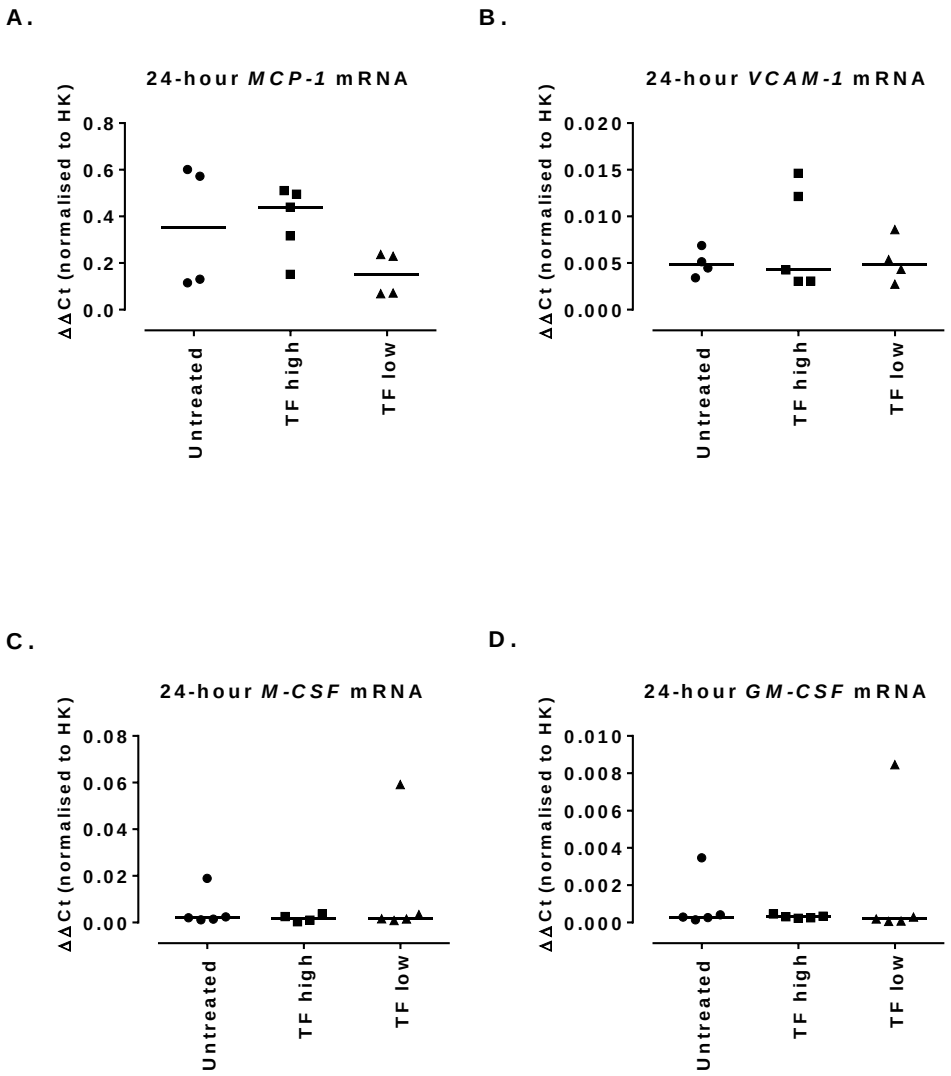


Figure 5.7 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with TF high and low concentrations.

A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=4/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.

5.4.2.4.The effect of L-PGDS on the pro-inflammatory gene expression

The basal levels of pro-inflammatory cytokine and chemokine mRNA expression were not altered either by L-PGDS high (1,200 ng/ml) or L-PGDS low (300 ng/ml) 24-hour treatments. More specifically, no statistically significant changes were observed for *IL-6* (**Figure 5.8-A**), *IL-8* (**Figure 5.8-B**), *MIP-1α* (**Figure 5.8-C**) and *IP-10* mRNA levels (**Figure 5.8-D**) after CP treatments when compared to the expression levels in untreated ciGEnCs.

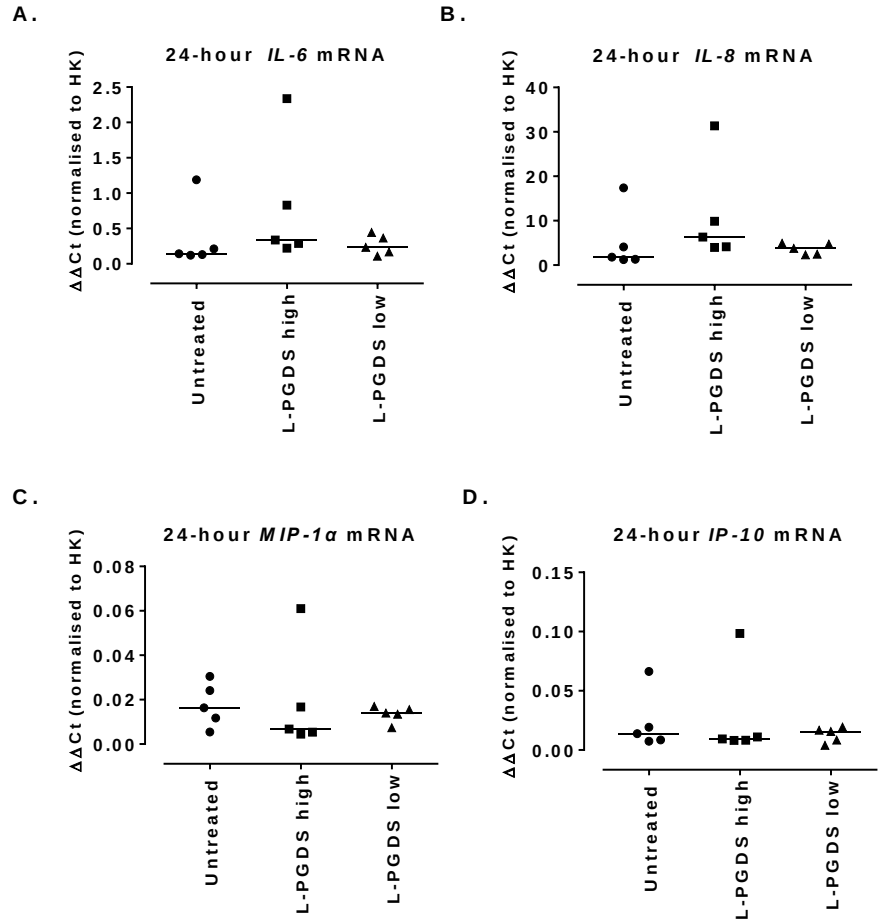


Figure 5.8 qRT-PCR for cytokine and chemokine expression after 24-hour treatments with L-PGDS high and low concentrations.

A. Changes in IL-6 mRNA levels. B. Changes IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes IP-10 mRNA levels. N=5/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS vs untreated. HK: ACTB, TBP, TUBB.

The basal levels of pro-inflammatory urinary biomarker and blood cell growth factor mRNA expression were not altered either by L-PGDS high or L-PGDS low 24-hour treatments. More specifically, no statistically significant changes were observed for *MCP-1* (**Figure 5.9-A**) or *VCAM-1* (**Figure 5.9-B**) after L-PGDS treatments when those were compared to the untreated ciGEnCs. Similarly, the L-PGDS high and low concentrations were not able to modulate the basal mRNA expression levels of *M-CSF* (**Figure 5.9-A**) and *GM-CSF* (**Figure 5.9-B**) in ciGEnCs.

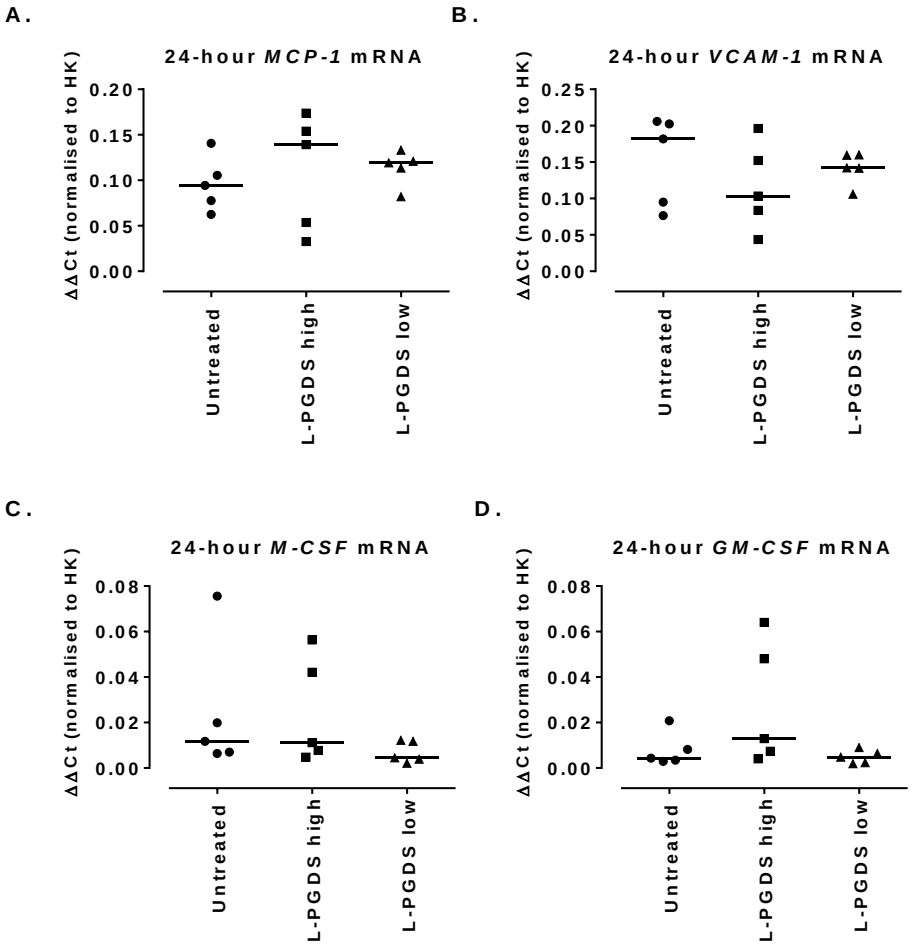


Figure 5.9 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with L-PGDS high and low concentrations.

A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=4/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.

5.4.2.5.The effect of MCP-1 on the pro-inflammatory gene expression

The basal mRNA expression levels of cytokines and chemokines (expressed by the untreated ciGEnCs) were not modified either by MCP-1 high (0.26 ng/ml) or MCP-1 low (0.172 ng/ml) 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of TF high and low concentration treatments on *IL-6* (**Figure 5.10-A**), *IL-8* (**Figure 5.10-B**), *MIP-1α* (**Figure 5.10-C**) *and IP-10* (**Figure 5.10-D**) were compared to the untreated ciGEnCs.

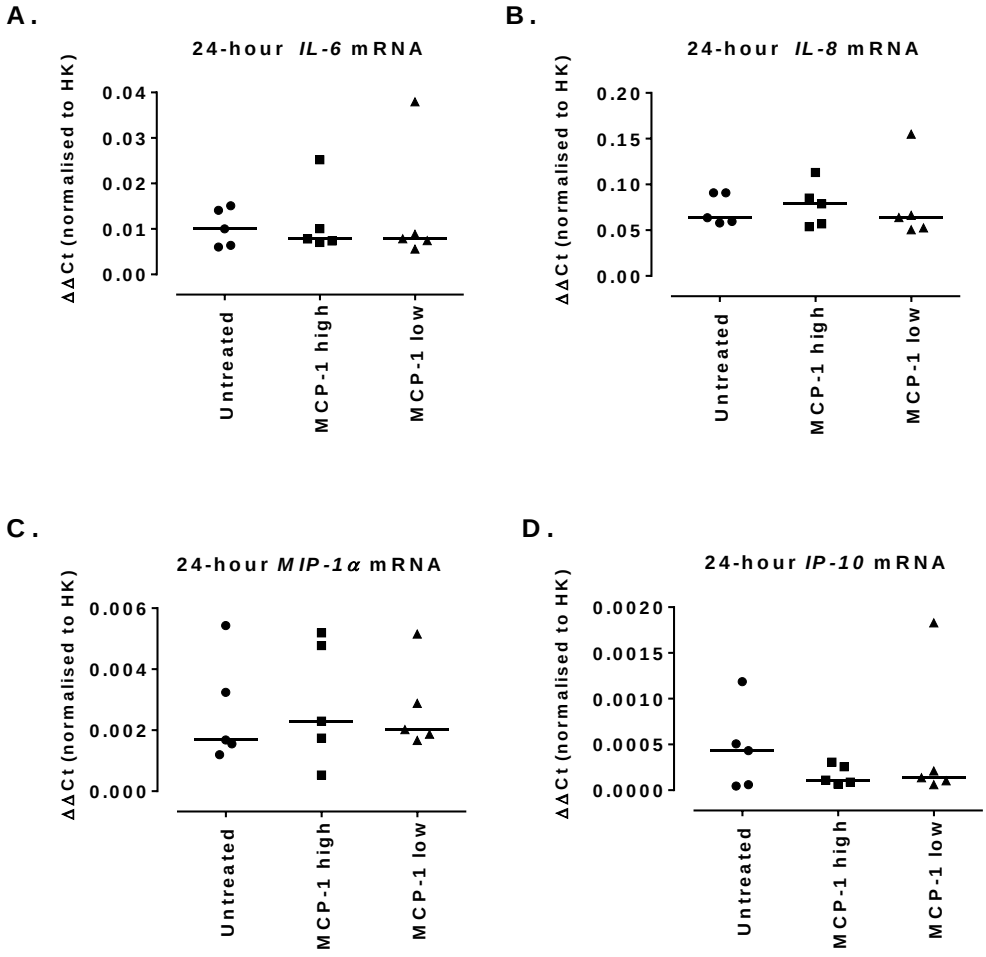


Figure 5.10 qRT-PCR for cytokine and chemokine expression after 24-hour treatments with MCP-1 high and low concentrations.

A. Changes in IL-6 mRNA levels. B. Changes IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes IP-10 mRNA levels. N=5/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS vs untreated. HK: ACTB, TBP, TUBB.

The mRNA levels of urinary biomarkers expressed by the untreated ciGEnCs were not affected either by MCP-1 high or MCP-1 low 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of MCP-1 high and low concentration treatments on *MCP-1* (**Figure 5.11-A**) and *VCAM-1* (**Figure 5.11-B**) were compared to the untreated ciGEnCs.

The mRNA expression levels of *M-CSF* were statistically significantly increased after MCP-1 high treatments (0.001 [0.001-0.002], p=0.02) compared to MCP-1 low treatments (0.0009 [0.0009-0.001]). Although they were not statistically significantly higher of those of untreated ciGEnCs (0.001 [0.0007-0.001]), the *M-CSF* levels of the latter were, in general, lower than those induced by the MCP-1 high concentrations (**Figure 5.11-C**). The MCP-1 high and low concentrations did not significantly change the expression of *GM-CSF* (**Figure 5.11-D**).

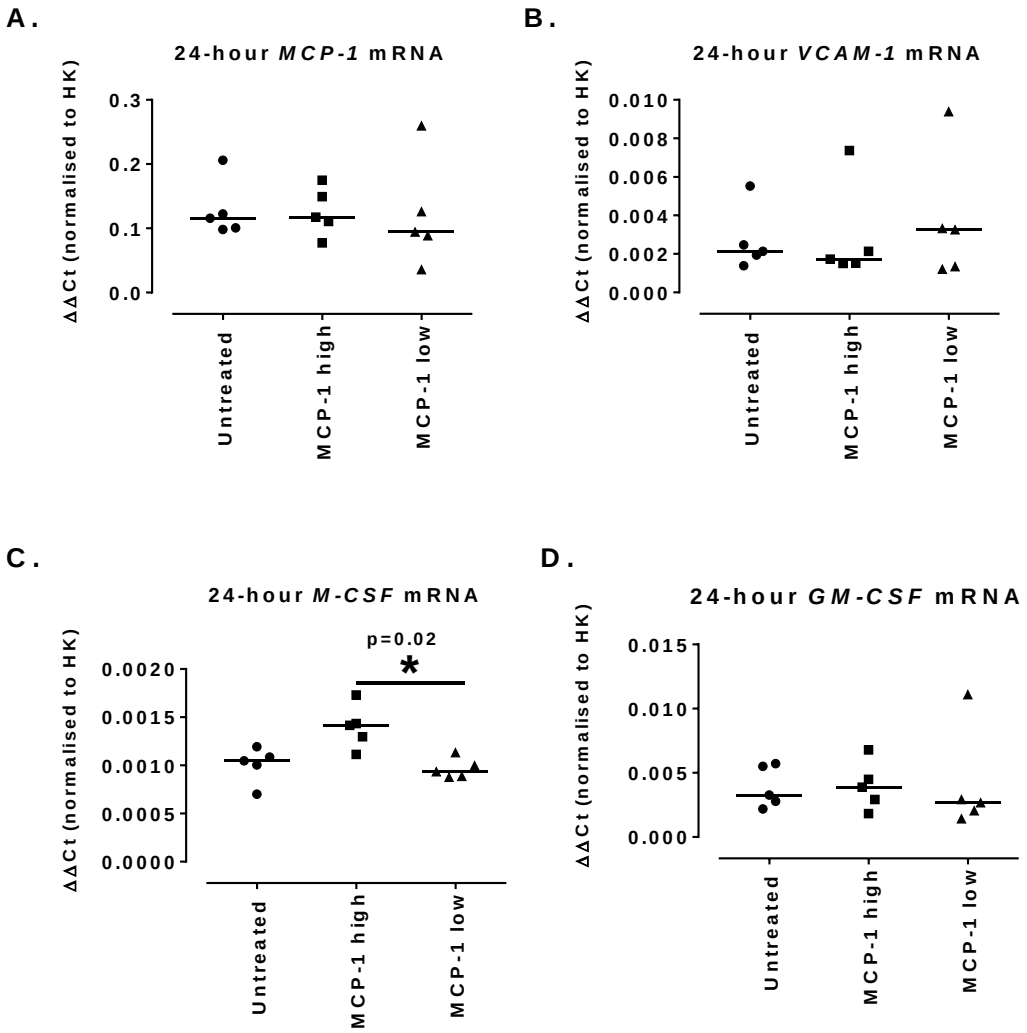


Figure 5.11 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with L-PGDS high and low concentrations.

A. *Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=4/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.*

5.4.2.6.The effect of NGAL on the pro-inflammatory gene expression

The baseline mRNA levels of pro-inflammatory cytokines and chemokines expressed by the untreated ciGEnCs were not altered either by NGAL high (34 ng/ml) or NGAL low (22.2 ng/ml) 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of AGP high and low concentration treatments on *IL-6* (**Figure 5.12-A**), *IL-8* (**Figure 5.12-B**), *MIP-1α* (**Figure 5.12-C**) and *IP-10* mRNA levels (**Figure 5.12-D**), were compared to the basal expression levels in untreated ciGEnCs.

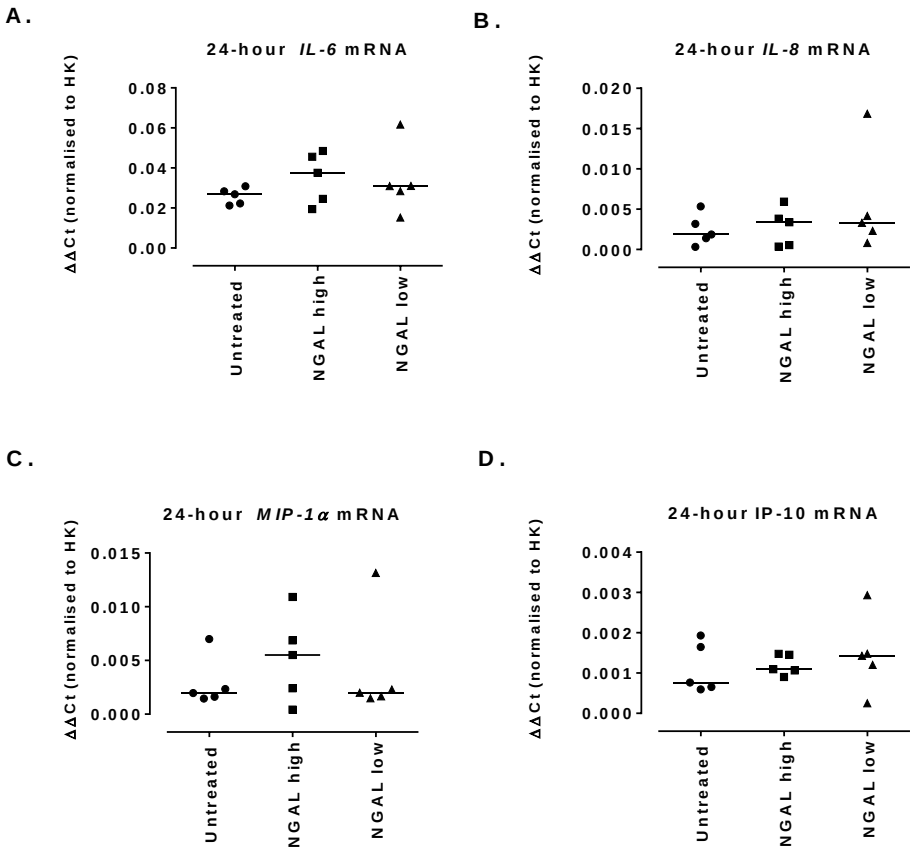


Figure 5.12 qRT-PCR for cytokine and chemokine expression after 24-hour treatments with NGAL high and low concentrations.

A. Changes in IL-6 mRNA levels. B. Changes IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes IP-10 mRNA levels. N=5/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS vs untreated. HK: ACTB, TBP, TUBB.

The baseline mRNA levels of urinary biomarkers and blood cell growth factors expressed by the untreated ciGEnCs were not altered either by AGP high or AGP low 24-hour treatments. More specifically, no statistically significant changes were observed when the effects of AGP high and low concentration treatments on *MCP-1* (**Figure 5.13-A**), *VCAM-1* (**Figure 5.13-B**), *M-CSF* (**Figure 5.13-C**) and *GM-CSF* (**Figure 5.13-D**) were compared to the basal expression levels in untreated ciGEnCs.

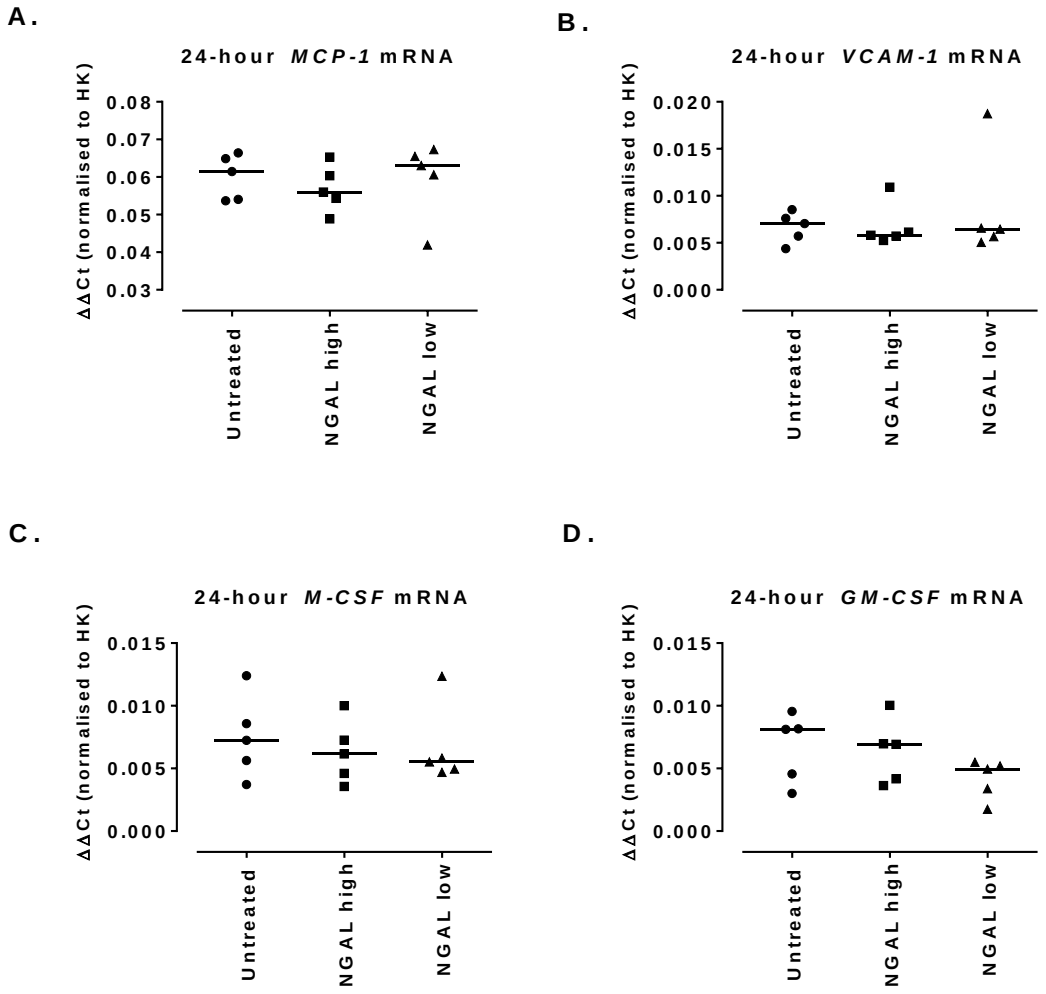


Figure 5.13 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with NGAL high and low concentrations.

A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=4/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.

5.4.3. The combined biomarker treatment did not have a prominent effect on pro-inflammatory gene mRNA expression

Following assessing the effect of individual biomarker treatments, the effect of combined 24-hour biomarker treatments (AGP, CP, L-PGDS, TF, MCP-1, NGAL) at high and low concentrations was assessed, as *in vivo* the biomarkers could be simultaneously present within the glomerulus upon renal inflammation. After the 24-hour ciGEnC incubations, potential changes in the mRNA expression of cytokines, chemokines, urinary biomarkers, blood cell growth factors (same cytokines, chemokines, urinary biomarkers and blood cell growth factors that were tested on Chapters 3 and 4). In addition, with TNF-α and IL-1β having the most robust effect on *in vitro* ciGEnC activation among all the cytokines tested in Chapter 3, potential changes in their receptors, were investigated.

5.4.3.1. The effect of combined biomarker treatments on ciGEnC cytokine and chemokine mRNA expression

IL-6 mRNA expression was significantly increased after the biomarker low concentration treatments (0.4 [0.3-0.9], p=0.05) but not after the biomarker high concentration treatments (0.3 [0.3-0.7]) when compared to the untreated ciGEnCs (0.2 [0.03-0.4]) (**Figure 5.14-A**). No statistically significant differences were observed for *IL-8* (**Figure 5.14-B**), *MIP-1α* (**Figure 5.14-C**) and *IP-10* (**Figure 5.14-D**) mRNA expression after combined biomarker treatments (high and low) when those were compared to the baseline expression levels of the untreated ciGEnCs.

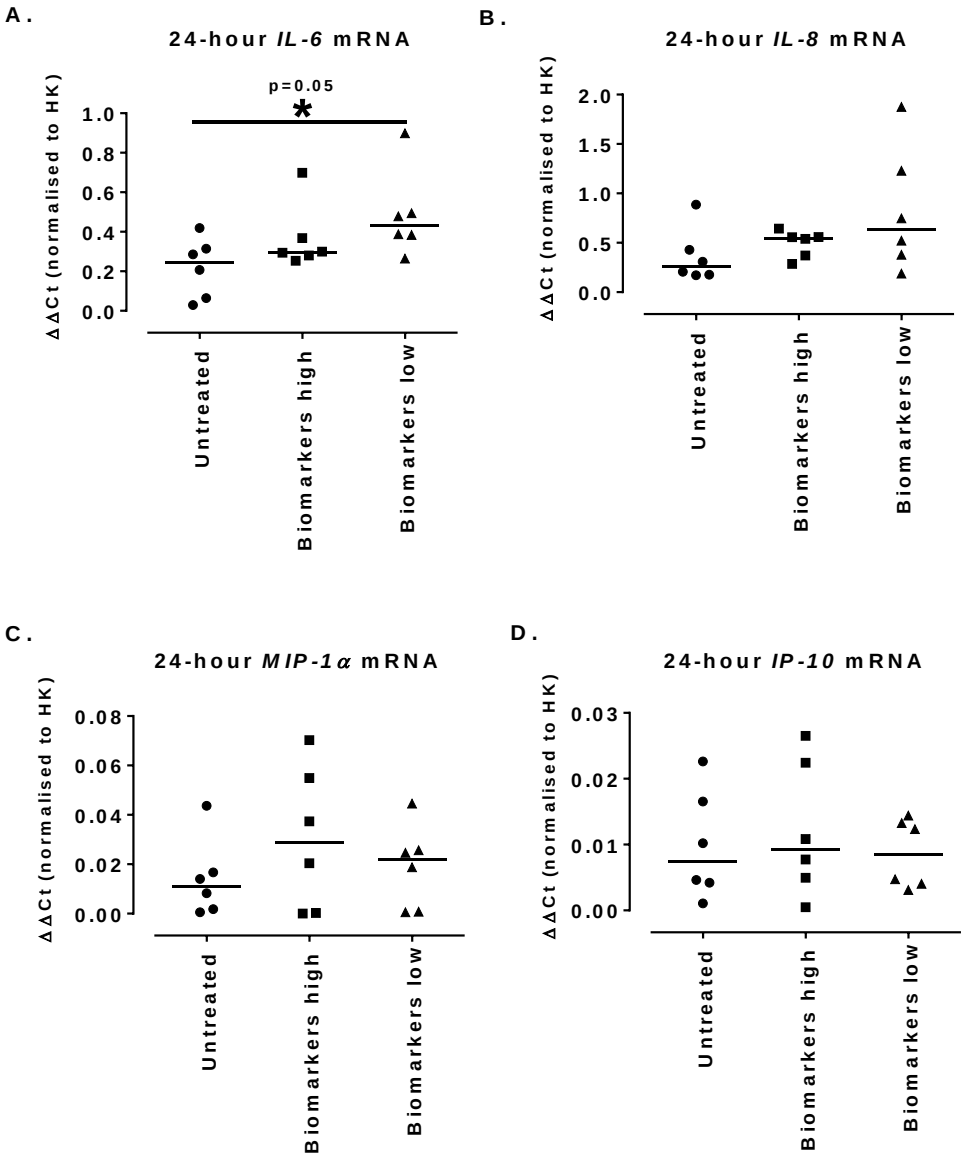


Figure 5.14 qRT-PCR for cytokine and chemokine expression after 24-hour treatments with combination of biomarkers.

*A. Changes in IL-6 mRNA levels. B. Changes IL-8 mRNA levels. C. Changes in MIP-1α mRNA levels. D. Changes IP-10 mRNA levels. N=6/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05 vs untreated. HK: ACTB, TBP, TUBB.*

5.4.3.2.The effect of combined biomarker treatments on ciGEnC blood cell growth factor mRNA expression

The combined biomarker treatments did not have a prominent effect on urinary biomarker mRNA expression. More specifically, no statistically significant changes were observed when the combined biomarker high and low concentration treatments on *MCP-1* (**Figure 5.15-A**) and *VCAM-1* (**Figure 5.15-B**) mRNA were compared to the untreated ciGEnCs. Similarly, the combined biomarker high and low concentrations did not significantly change the expression of *M-CSF* (**Figure 5.15-C**) and *GM-CSF* (**Figure 5.15-D**) when compared to the untreated ciGEnCs.

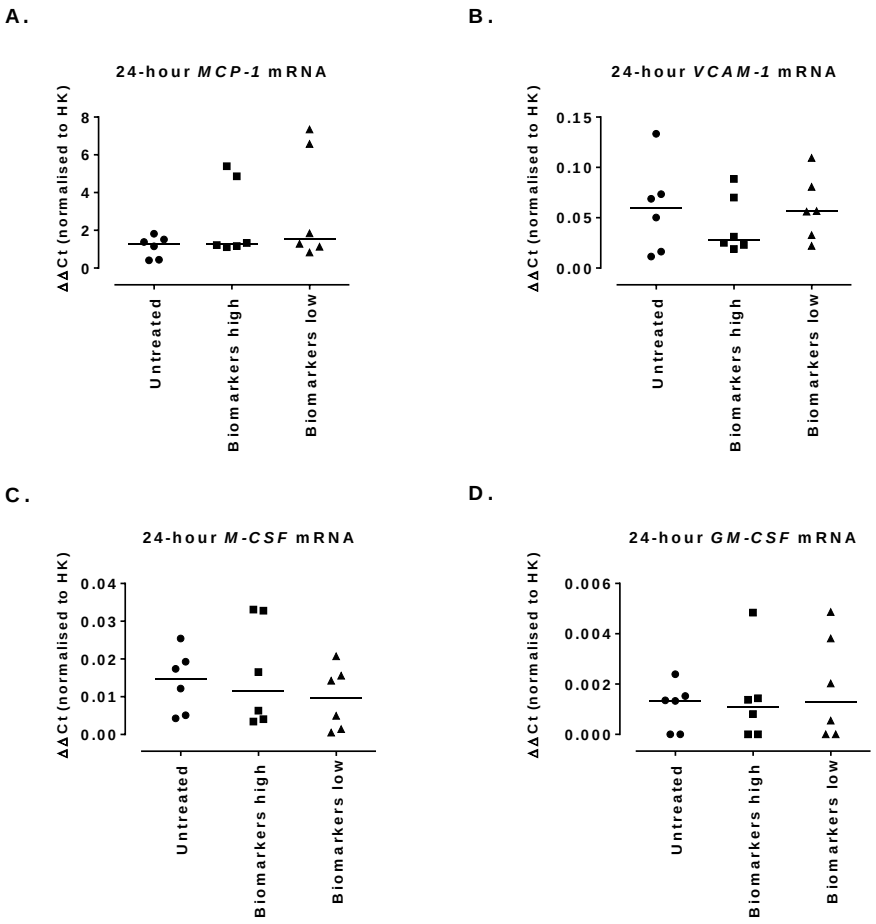


Figure 5.15 qRT-PCR for pro-inflammatory urinary biomarker and blood cell growth factor expression after 24-hour treatments with combination of biomarkers.

A. Changes in MCP-1 mRNA levels. B. Changes in VCAM-1 mRNA levels. C. Changes in M-CSF mRNA levels. D. Changes in GM-CSF mRNA levels. N=6/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS. HK: ACTB, TBP, TUBB.

5.4.3.3.The effect of combined biomarker treatments on ciGEnC cytokine receptor mRNA expression

TNFR1 (**Figure 5.16-A**) and *TNFR2* (**Figure 5.16-B**) mRNA expression was not modulated by high or low concentration combined biomarker treatments compared to untreated ciGEnCs. Similarly, *IL-1RI* mRNA expression was also unaffected (**Figure 5.16-C**). *IL-1RII* mRNA expression, however, was statistically significantly increased

after high-concentration combined biomarker treatments (0.004 [0.003-0.01], p=0.05) compared to the untreated ciGEnCs (0.002 [0.0005-0.009]) (**Figure 5.16-D**).

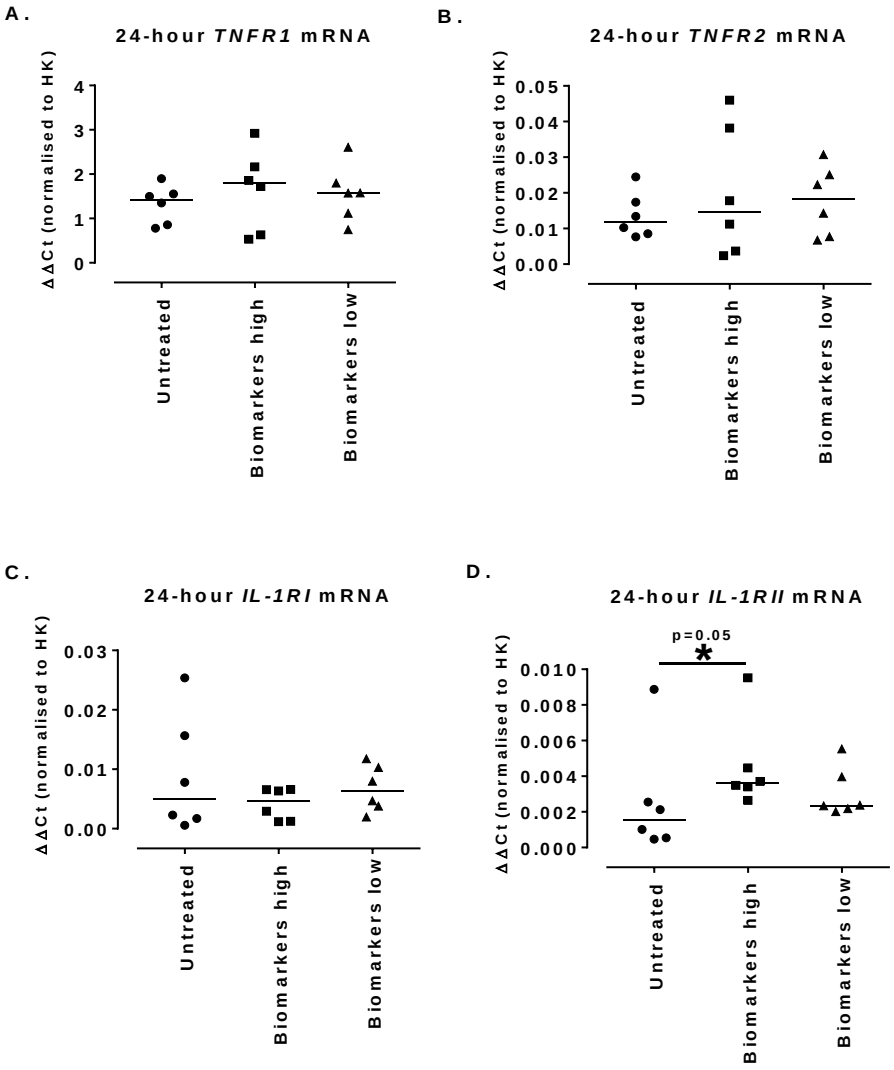


Figure 5.16 qRT-PCR for TNF-α and IL-1 recpetor expression after 24-hour treatments with combination of biomarkers.

*A. Changes in TNFR1 mRNA levels. B. Changes in TNFR2 mRNA levels. C. Changes in IL-1RI mRNA levels. D. Changes in IL-1RII mRNA levels. N=6/group. Data presented as median ΔΔCt, are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *p≤0.05. HK: ACTB, TBP, TUBB.*

5.4.4. The combined biomarker treatment did not have a prominent effect in pro-inflammatory protein secretion

No remarkable changes on pro-inflammatory gene expression were observed after the 24-hour combined high or low concentration biomarker treatments. However, as gene expression alone could not necessarily reflect changes in the protein level, the potential effect of 24-hour combined biomarker treatments on pro-inflammatory protein secretion (cytokines, chemokines, urinary biomarkers, blood cell growth factors) was also tested via ELISA, using ciGEnC conditioned media.

5.4.4.1.The effect of combined biomarker treatments on ciGEnC cytokine secretion

Potential changes in TNF-α, IFN-γ, IL-6 and IL-10 secretion were tested. However, neither the high nor the low biomarker concentrations induced any changes in the secretion of TNF-α (**Figure 5.17-A**), IFN-γ (**Figure 5.17-B**), IL-6 (**Figure 5.17-C**) or IL-10 protein (**Figure 5.17-D**).

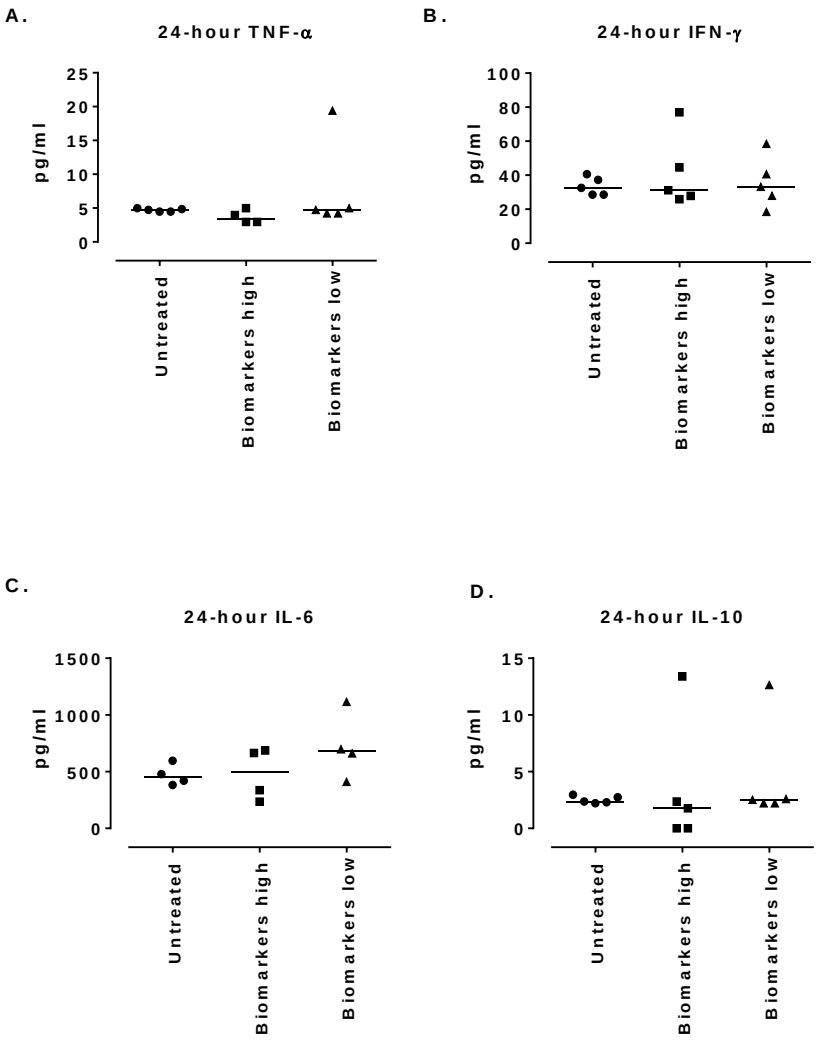


Figure 5.17 Cytokine secretion after 24-hour ciGEnC-stimulation combined biomarker treatments.

A. TNF-α. B. IFN-γ. C. IL-6. D. IL-10. N=4-5/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS.

5.4.4.2.The effect of combined biomarker treatments on ciGEnC chemokine secretion

Potential changes in IL-8, MIP-1α and IP-10 secretion were tested. However, neither the high nor the low biomarker concentrations induced any changes in the secretion of IL-8 (**Figure 5.18-A**), MIP-1α (**Figure 5.18-B**) or IP-10 (**Figure 5.18-C**).

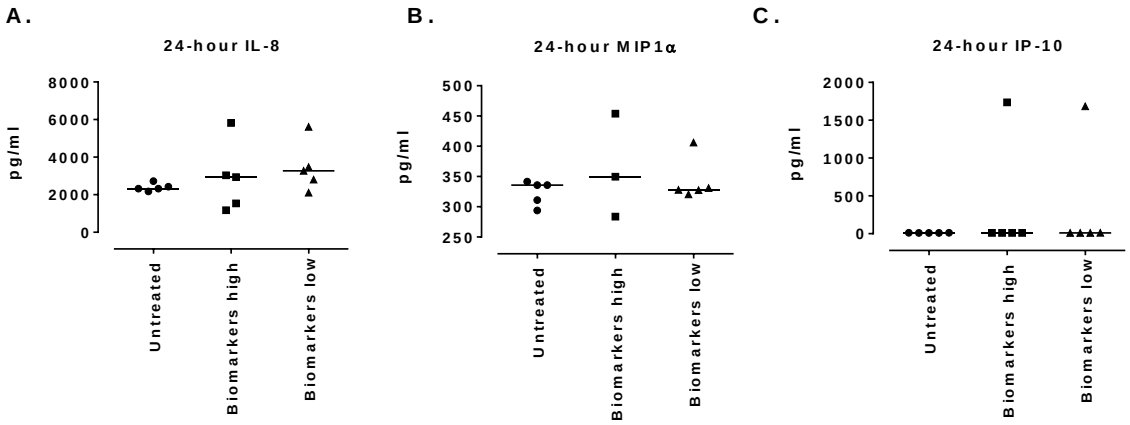


Figure 5.18 Chemokine secretion after 24-hour ciGEnC-stimulation combined biomarker treatments.

A. IL-8. B. MIP-1α. C. IP-10. N=4-5/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS.

5.4.4.3.The effect of combined biomarker treatments on ciGEnC blood cell growth factor secretion

Potential changes in sVCAM-1, M-CSF and GM-CSF secretion were also investigated. However, neither the high nor the low biomarker concentrations induced any changes in the secretion of sVCAM-1 (**Figure 5.19-A**), M-CSF (**Figure 5.19-B**) or GM-CSF (**Figure 5.19-C**).

The low biomarker treatments led to a significantly lower secretion of sVCAM-1 (72 pg/ml [27-154], p=0.03) compared to secretion in the untreated ciGEnCs (189 pg/ml [170-212]) (**Figure 5.19-A**). M-CSF (**Figure 5.19-B**) and GM-CSF (**Figure 5.19-C**) secretion, however, remained unaffected.

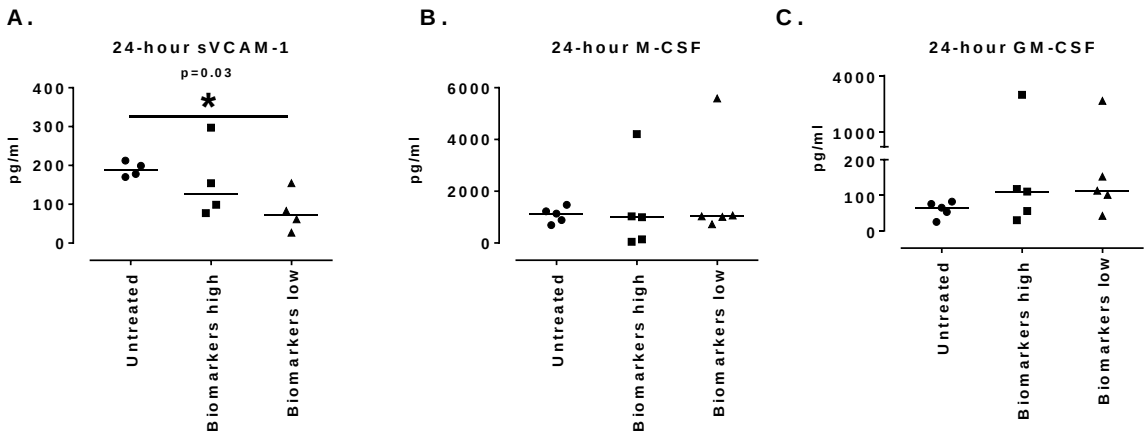


Figure 5.19 Urinary biomarker and blood cell growth factor secretion after 24-hour ciGEnC-stimulation combined biomarker treatments.

*A. sVCAM-1. B. M-CSF. C. GM-CSF. N=4-5/group. Data presented as median concentrations are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *P≤0.05.*

5.4.5. The combined biomarker treatment did not have a prominent effect in surface adhesion molecule expression

Potential changes in the surface expression of the adhesion molecules VCAM-1 and ICAM-1 were investigated after incubations of ciGEnCs for 24-hour with a combination of biomarkers at high or low concentrations.

However, neither the high nor the low biomarker concentration treatments induced VCAM-1 surface expression by the ciGEnCs nor modified the basal surface ICAM-1 levels, constitutively expressed by the untreated ciGEnCs **(Figure 5.20**).

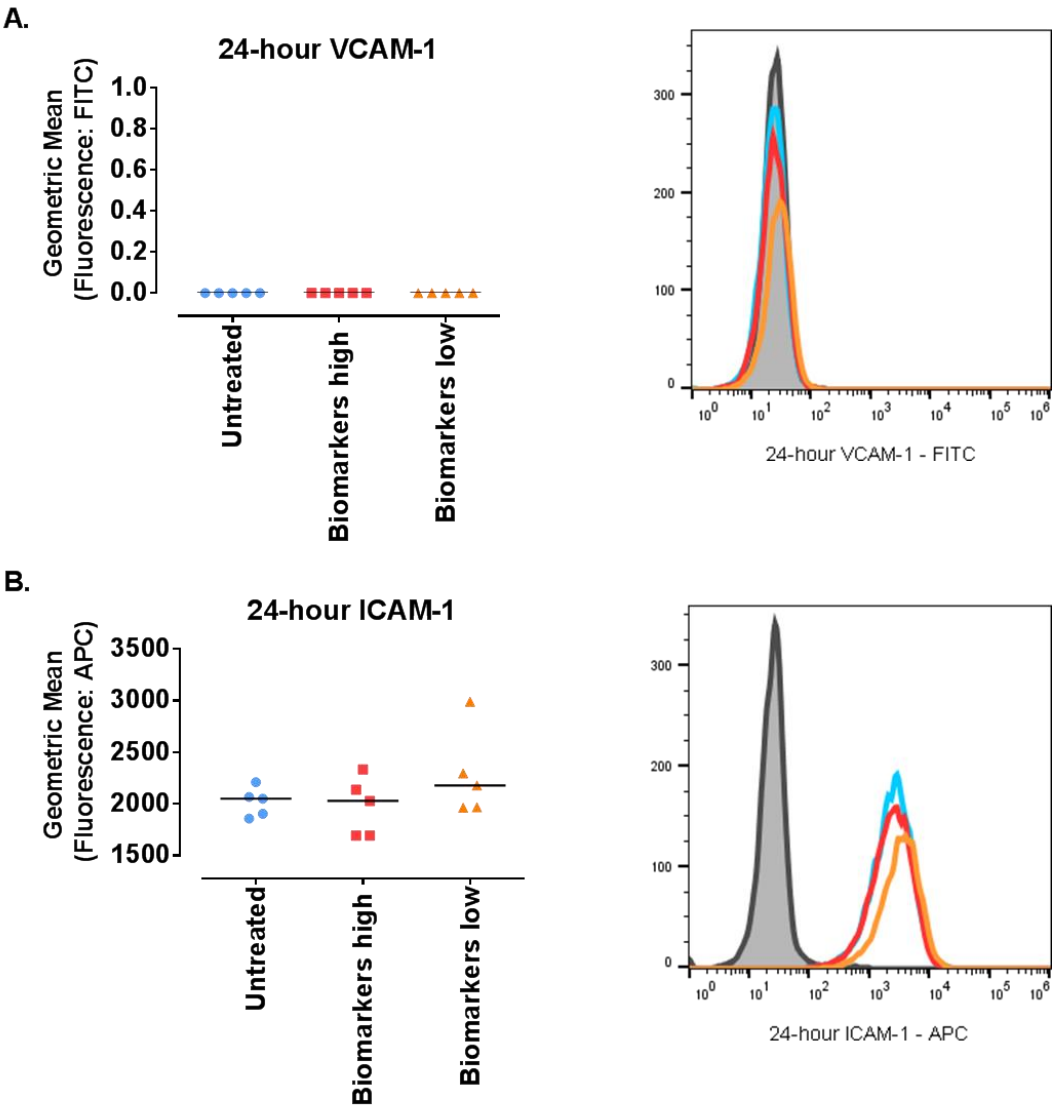


Figure 5.20 VCAM-1 and ICAM-1 surface expression following 24-hour stimulation with combined biomarker treatments.

A. 24-hour VCAM-1 surface expression. B. 24-hour ICAM-1 surface expression. N=4-5/group. Data presented as (median [range]) are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS.

5.4.6. The combined biomarker treatment did not induce increased cellular death

The apoptotic/necrotic levels of ciGEnCs after 24-hour of incubation with all biomarkers at either high or low concentrations were tested.

The majority of ciGEnCs remained viable (Anx V-/PI-) after 24-hour treatment with high-concentration or low-concentration biomarkers, similarly to the untreated ciGEnCs (**Figure 5.21-A & B**). No significant changes were observed in the levels of apoptotic (Anx V+/PI-) and late apoptotic/necrotic (Anx V+/PI+) ciGEnCs among the different treatments (**Figure 5.21-A & B**).

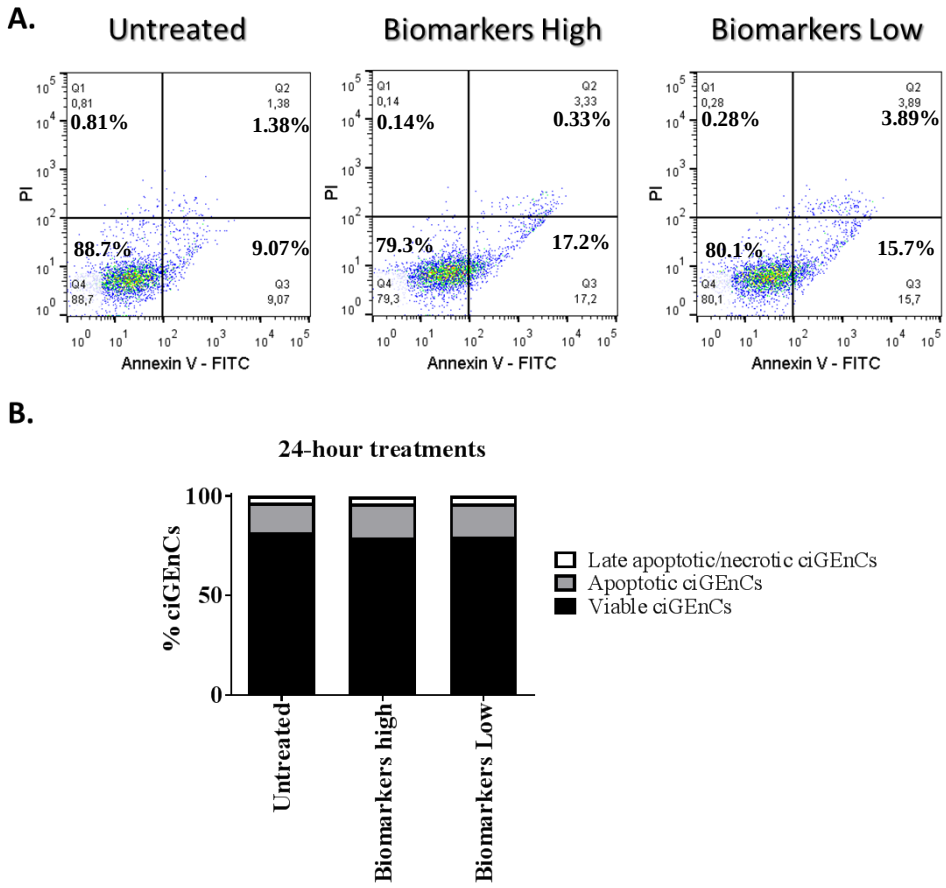


Figure 5.21 ciGEnC viability after 24 hours of combined biomarker treatments.

A. Flow cytometric plots of Annexin V-FITC/PI-stained untreated ciGEnCs and ciGEnCs treated for 24 hours with biomarker high and low concentrations. B. Diagrams of viable (Anx V-/PI-), early apoptotic (Anx V+/PI-) and late apoptotic/necrotic (Anx V+/PI+) ciGEnCs after 24 hours of biomarker high and low concentration treatments. N=3/group.

5.5. Discussion

This chapter aimed at investigating the potential role of the human urinary biomarkers on the human GEnCs. Although experimentally validated in a UK and two international cohorts of children with JSLE in our laboratory using patient urine samples[452], these biomarkers are not currently used in clinic as routine diagnostic tools. In the future, however, they could prove to be an essential tool for routine monitoring of young JSLE patients' renal disease activity. The ciGEnCs were previously shown to substantially express and produce only two of the human urinary biomarkers for LN, MCP-1 and VCAM-1, especially under pro-inflammatory stimulation (**Chapter 3**). Thus, the human GEnCs are not the main source of urinary biomarker production.

For this reason, primary human monocytes were isolated, cultured *in vitro* and differentiated into macrophages and then were stimulated for 48 hours with IFN-γ or LPS to promote an M1-like phenotype. The human macrophages were found to express mRNA for all urinary biomarkers, except for *NGAL*. mRNA expression for *AGP*, *CP*, *MCP-1* and *VCAM-1* was considerably increased either after IFN-γ or after LPS stimulation, whereas the macrophage-expressed *TF* and *L-PGDS* basal levels were not altered by IFN-γ and LPS. The intra-renal presence of macrophages could therefore be an important source of urinary biomarkers within the nephritic kidneys. Thus, upon a renal flare, the kidney-recruited macrophages could stimulate and/or secrete the biomarkers within the renal microenvironment affecting, potentially, the human GEnCs and other glomerular components.

The next step of this study was to treat *in vitro* the human ciGEnCs with human recombinant biomarkers individually or in combination, at either high or low concentrations. The high and low levels of biomarkers used for the ciGEnCs treatments

represent the “concentrated” levels of the biomarkers, after GFB filtration, as found in the urine of patients with active and inactive LN. They did not necessarily represent the intra-renal biomarker levels. Thus, it could be argued that as the human GEnCs are the first component of the GFB to encounter blood circulating factors, the LN biomarkers would be present in much less concentrated levels within the blood circulation by the time of their GEnC interaction. Consequently, potential changes observed in ciGEnCs after biomarker high/low treatments, could be attributed to abnormally high concentrations used and not to *in vitro* imitation of a physiological effect. It needs to be noted, however, that both the high and low biomarker treatments ranged among low microgram and predominantly between nanogram and picogram concentrations and as these concentrations are low, no further biomarker dilutions (lower concentrations) were used to treat the ciGEnCs *in vitro*. The lack of an *in vitro* effect of combined low-concentration biomarker treatments on ciGEnCs, however, does not imply the lack of an i*n vivo* effect on the human GEnCs.

Furthermore, neither the high nor the low urinary biomarker concentrations, either when used to treat the cells individually or when used all together in combination, had any remarkable effect on the ciGEnCs. In general, apart from inducing *IL-6* mRNA expression, the combined low biomarker treatments were not found to have any effect on the gene expression, protein secretion or surface expression of pro-inflammatory proteins tested. In a similar manner, apart from inducing *IL-1RII* mRNA expression, the combined high biomarker treatments neither reinforced the expression and production of pro-inflammatory proteins by the ciGEnCs nor induced a proportionate increase in the protein levels in comparison to the low biomarker treatments. In addition, the low and high biomarker treatments had no effect on ciGEnC viability in terms of apoptosis/necrosis levels.

The lack of urinary biomarker effect on human ciGEnCs might be attributed to several reasons. First, the human GEnCs might lack expression of some of the biomarker receptors and thus they could be unresponsive to the potential effect of the biomarkers. This possibility was not tested in this thesis project. For, example human brain endothelial cells of patients that underwent brain scans with positron emission tomography[453] but also *in vitro* cultured human brain endothelial cells [454] have been found to express TF receptors. In rats, the liver endothelium has been shown to be responsible for CP uptake and internalisation via surface receptors for CP; subsequently the liver endothelium modifies CP and mediates its release and uptake by the hepatocytes[455].

Megalin or low-density lipoprotein-related protein 2 (LPR2), the receptor of NGAL[456], is expressed by the human proximal tubular epithelial cells in the kidney[457] and also by the human renal podocytes[458], however the human endothelium does not seem to express megalin. HUVECs and human dermal microvascular endothelial cells (HMECs) have been shown to express CCR2, the receptor of MCP-1[459] whereas AGP, also known as ORM1, has the ability to bind glycoproteins of the endothelial cell glycocalyx, regulating the permeability of the endothelium[12]. Finally, a comprehensive literature search did not reveal any studies reporting the potential effect of L-PGDS binding onto human endothelial cells, however it has to be noted that L-PGDS expression itself on the vascular endothelium has been shown to be induced by fluid shear stress[460]. It is therefore possible that lack of increased expression levels of *L-PGDS* mRNA by the ciGEnCs after either cytokine/LPS or serum incubations as shown Chapters 3 and 4 respectively, could be attributed to the fact that no appropriate stimuli were used to induce L-PGDS expression.

Future studies on ciGEnCs could therefore test the surface expression of urinary biomarker receptors, especially megalin, CCR2 and TF receptors, including both basal expression levels as displayed by untreated ciGEnCs but also potential alterations of receptor expression levels following pro-inflammatory stimulation. Furthermore, the dynamics of AGP-ciGEnC glycocalyx binding could also provide interesting findings in terms of AGP-mediated regulation of human GEnC function.

It should also be highlighted that ciGEnCs were exposed to biomarkers for only one time period (24 hours) and thus a more acute (2-12 hours) or chronic (48-96 hours) effect on the ciGEnCs could have been missed. For this reason, future studies could introduce time-response assays for individual and combined biomarker high and low concentrations, this time also including VCAM-1 (although in contrast to MCP-1, no literature evidence was found for VCAM-1 effect in pro-inflammatory protein expression, viability or other functions of the human endothelium). In addition, higher *in vitro* concentrations of the biomarkers could have a more robust effect on ciGEnCs. For example, 12- and 24-hour incubations of HUVECs with 1μg/ml of human recombinant NGAL have been shown to induce a statistically significantly higher increase in the secreted levels of IL-6, IL-8 and MCP-1 compared to untreated HUVECs[461].

Moreover, the human urinary biomarkers could affect the human renal glomerular endothelium in different ways than modifying pro-inflammatory protein expression and reducing their viability, such as inducing changes in the permeability of GEnCs and GFB-related filtering capacity or modifying the glycocalyx structure of GEnCs. Indeed, HMECs from dermal tissue have been shown to express AGP, in contrast to ciGEnCs, and AGP is necessary for the capillary charge-selectivity barrier[462]. AGP has also been shown to have an anti-inflammatory effect on HUVECs via reducing their response to

histamine-mediated upregulation of metabolic pathways leading to increased acidification rates[463]. In addition, AGP has been shown to inhibit *in vitro* angiogenesis as it was able to block the formation of capillary-like tubes by human lung microvascular endothelial cells as well as endothelial cell adhesion to ECM and migration in wound healing assays[464]. Similarly, MCP-1 has been shown to promote angiogenesis on human aortic endothelial cells via inducing VEGFA[465]. Furthermore, MCP-1 has been found to have a chemotactic effect on HUVECs and human dermal microvascular endothelial cells (HMECs)[459].

In addition to the reasons of lack of biomarker effect listed above, it is important to also consider another possibility. The biomarkers could be acting on the human glomerular endothelium of the kidney only in conjunction with pro-inflammatory cytokines such as TNF-α and IL-1β leading to "priming" of the endothelium. Alternatively, they could be using the cytokines as an "anchor" to bind to the cytokine receptors and potentially get internalised within the GEnCs. Henceforth, it is important that future studies use cytokines known to have an effect on human ciGEnCs, such as TNF-α, IL-1β, IL-13 and IFN-γ as shown on **Chapter 3**, to treat the cells along with the human recombinant biomarkers, at high or low concentrations, to investigate potential joint effects of cytokines and biomarkers.

Finally, another possible explanation could be that the human urinary biomarkers for LN have no direct or indirect effect on human GEnCs. Instead, upon LN renal inflammation, immune cells such as human macrophages could be activated to produce and release the biomarkers locally in the kidney as acute-phase proteins or "alarm signals". Continuing inflammation and lack of resolution of inflammation, could eventually lead to chronic renal disease and GFB damage and thus, as a bystander effect of GEnC (as well as podocyte and GBM) destruction, passive release of the human

urinary biomarkers could occur. The levels of the biomarkers present within the patients' urine, could then be reflective of the extent of kidney damage, but without having themselves a role on the destruction of the glomerular renal structures.

5.6. Conclusion

In conclusion, the human ciGEnCs are not induced to produce pro-inflammatory proteins *in vitro* following treatments with high and low concentrations of the novel urinary biomarkers for active LN. No apoptosis or necrosis are observed under the influence of these biomarkers.

6. Chapter 6: General Discussion

6.1. Aims of the thesis

The overarching aim of this thesis was to study in-depth the role of human GEnCs in LN inflammation to test the hypothesis that human GEnCs are not just passive bystanders but are actively involved in the development and perpetuation of renal inflammation in LN.

An initial in-depth literature review did not identify a robust volume of experimental work on the pro-inflammatory activity of human GEnCs, indeed it identified a striking paucity of relevant data. In contrast to other types of human endothelial cells, such as HUVECs and human brain endothelial cells[345,466], the ability of the human GEnCs to express and produce pro-inflammatory proteins under different pro-inflammatory stimuli had not been extensively studied. Moreover, in both adult-onset disease, but particularly in JSLE, the pro-inflammatory action of human GEnCs in the context of the inflammatory environment of the LN disease model had not been previously studied thoroughly.

For all the above reasons and considering the important position of human GEnCs at the blood-renal tissue interface as well as their role as part of the GFB[1], the focus of this thesis was to develop a number of different *in vitro* models of LN using human ciGEnCs so as to study different aspects of GEnC involvement in renal disease. Taking into consideration the possible interactions between the human GEnCs and other renal cells, blood circulating factors and immune cells, the *in vitro* ciGEnC models were used to create, as far as was possible within the limitations of an *in vitro* environment, a holistic picture of the human GEnC involvement in renal inflammation. To achieve these

goals, the human ciGEnCs were cultured and were exposed to various potential pro-inflammatory stimuli, as presented and described in each of the **Chapters 3**, **4** and **5**.

6.2. Summarising the findings from ciGEnC experiments and their importance in the context of lupus nephritis

Initially, the ciGEnCs were incubated for 15 minutes, 30 minutes, 4 hours and 24 hours with cytokines known to be involved in LN (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-13, VEGF) and with LPS; the production of pro-inflammatory proteins, the activation of pro-inflammatory transcription factors as well as their survival after 24-hour exposure to cytokines/LPS were then tested.

The human ciGEnCs remained viable after cytokine/LPS exposure. They underwent NF-κB and STAT-1 activation and produced a variety of pro-inflammatory proteins due to stimulation induced by four out of the seven cytokines tested (TNF-α, IL-1β, IL-13 and IFN-γ) and LPS. These included; cytokines, chemokines, blood cell growth factors, urinary biomarkers, adhesion and co-stimulatory molecules. Importantly, all proteins produced by the ciGEnCs after cytokine/LPS stimulation have been previously associated with lupus pathogenesis and/or renal disease. These are all summarised in **Table 1.2** (and previously discussed in detail in Chapter 3).

It has been demonstrated that in a cohort of JSLE patients, 21% of whom had LN, IL-6 and IL-10 levels in the plasma of patients with active disease were significantly elevated compared to patients with inactive and compared to the plasma samples of HCs[312]. In ASLE, urinary IL-6 has been suggested to be an important marker of renal disease activity[467] whereas IL-10 serum levels have been found to be increased in SLE patients compared to HCs[468]. Furthermore in patients with type 2 diabetic nephropathy (DN), serum IL-6 levels have been found to be significantly elevated compared to serum samples from diabetic patients without nephropathy[469] whereas IL-10 polymorphisms have been strongly linked to type 2 DN susceptibility[470,471].

In ASLE patients with LN, significantly higher levels of urinary IL-8 have been observed compared to ASLE patients without LN and compared to HCs[472]. IL-8 serum levels have been shown to be significantly increased in patients with chronic renal failure compared to healthy individuals[473]. Urinary MCP-1 is one of the most widely-studied biomarkers for ASLE[474] and JSLE[234,326] renal disease activity. With regards to renal transplantation, patients who suffered acute rejection were found to have significantly elevated urinary levels of MCP-1 compared to those who were good responders to anti-rejection therapies[475].

MIP-1α has been found to be involved in the development of cellular crescents in human crescentic GN[215]. Furthermore, MIP-1α was found to be significantly enriched in the urine of LN patients compared to ASLE patients without LN[476]. Serum MIP-1α has also been found to be elevated in septic patients compared to healthy individuals in the serum of which MIP-1α was mostly undetectable[477]. In a study that included JSLE patients, 65.2% of which had LN- and HCs, the serum IP-10 levels were found to be

significantly elevated in JSLE patients with overall active disease but also in JSLE patients with active LN compared to those with inactive LN[478]. Furthermore, in a cohort of type I diabetic patients, urinary IP-10 levels were found to be significantly elevated in patients with microalbuminuria and early progressive deterioration of renal function compared to those with microalbuminuria and stable renal function as well as compared normoalbuminuric patients with stable renal function[479].

In a study with AAV patients, significantly higher levels of M-CSF have been detected in their plasma compared to HCs[480]; the more striking differences in M-CSF plasma levels were observed between AAV patients with LN and HCs[480]. Furthermore, conditionally immortalised human podocytes have been found to *in vitro* secrete increased M-CSF levels following pro-inflammatory stimulation[447]. GM-CSF local kidney production by MCs has been observed in GN patients and its levels have been associated to LN[213]. Neutrophils from hemodialysis and CKD patients incubated with GM-CSF *in vitro*, demonstrated improved survival and viability[481].

In JSLE, serum levels of a key pro-inflammatory cytokine, TNF-α, have been found to be significantly elevated in patients with active disease, in patients with LN and in patients with depression, compared to HCs[164]. In patients with DN, TNF-α serum and urinary levels have also been found to be significantly elevated compared to HCs[482,483]. In renal tissues of JSLE patients with LN significantly higher levels of positive IFN-γ staining have been observed compared to HCs[354]. In type II diabetic patients, significantly higher IFN-γ and TNF-α plasma levels have been observed in those with kidney disease compared to those without kidney disease[484].

Urinary VCAM-1 is an important biomarker for childhood LN and renal disease activity[234,236,326]. In patients of Asian origin with type II diabetes, plasma VCAM-1 has

been found to be associated with diabetic kidney disease[485]. In ASLE patients with LN significantly higher levels of serum ICAM-1 were detected compared to HCs[486]. Furthermore, ICAM-1 serum levels have been found to be significantly elevated in type II diabetic patients with microalbuminuria and are considered to be associated with diabetic kidney disease severity[487].

In ASLE patients, plasma ICOS-L was found to be significantly elevated in those with active disease compared to those with inactive disease or compared to patients with RA and furthermore, increased soluble ICOS-L levels inversely correlated with B cell ICOS-L surface expression levels[317]. In kidney biopsies from patients with DN and focal segmental GN significantly increased ICOS-L levels have been observed during early stages of the renal disease[488]; ICOS-L levels are then robustly decreased during later stages of the renal disease[488].

Monocyte PD-L1 surface expression in JSLE patients with active SLE have been found to be significantly reduced compared to HCs whereas patients in remission exhibited normal PD-L1 expression levels[374]. In a study using PBMCs from patients with proliferative and non-proliferative GN, the frequencies of PD-1/PD-L1-expressing CD4+ and CD8+ T cells have been found to be significantly increased compared to HCs, with the proliferative GN patients exhibiting even higher frequencies compared to the non-proliferative GN patients[489].

In this study, TNF-α and IL-1β have been identified as the most robust ciGEnC stimuli among the four cytokines found to have a profound effect on ciGEnC activation, leading to increased production of the majority of the pro-inflammatory proteins tested (MCP-1, VCAM-1, ICAM-1, IL-6, IL-8, M-CSF, GM-CSF, MIP-1α, ICOS-L).Following the findings presented in Chapter 3 and focusing on the significantly

increased production of pro-inflammatory proteins after cytokine/LPS stimulation, the target of all experimental assays performed and presented in Chapter 4 was to explore potential changes in the mRNA expression levels (and in the case of THP-1 cells in the protein secretion levels) of these pro-inflammatory proteins, as well as those of the TNF-α and IL-1β receptors).

Knowing that several of the cytokines used to stimulate the ciGEnCs were also found to be elevated in the plasma/serum of patients with active LN, serum samples from JSLE patients with active and inactive LN and serum samples from paediatric HCs were used to stimulate the ciGEnCs for 4 and 24 hours. Additional to the pro-inflammatory proteins, potential changes in the mRNA expression of fibrosis-related genes were also tested following the serum treatments. The JSLE serum treatments had a moderate to low effect in the expression of pro-inflammatory genes such as *IL-6* and *IL-1β* and of fibrosis-related genes such as *FN* and *LAMB2* and *COL1A1*.

Taking into account that JSLE patient sera may not be as effective on ciGEnC activation due to administration of immunosuppressive medications to the patients, the ciGEnCs were also treated with conditioned media of previously activated primary human neutrophils (TNF-α, IFN-γ, or LPS activation) and macrophages (IFN-γ or LPS activation). Additionally, a THP-1 macrophage cell line was used for experiments similar to those of primary human macrophages. The rationale behind these experiments was that upon renal inflammation, immune cells could infiltrate the glomerulus via the glomerular capillaries and once reaching there, they would secrete pro-inflammatory factors. Alternatively, immune cell-derived secreted factors could reach the glomerulus via the circulation, therefore they could be present in the patients' plasma/serum.

The primary human neutrophils did not particularly affect the ciGEnCs as compared to the primary human macrophages, with the latter being considered anyway as more potent pro-inflammatory cytokine producers. With the THP-1 cell providing a cell line of macrophages much more stable in terms of proliferation and survival compared to the primary human macrophages, it was possible to specifically study how the potential secretion of TNF-α and IL-1β after THP-1 stimulation could affect the human ciGEnCs *in vitro*. Indeed, blockade of TNF-α (via anti-TNF-α Ab) and IL-1β (via IL-1Ra) prior to addition of THP-1 conditioned media to the human ciGEnCs, abrogated (completely or partially) the THP-1 media-induced increased expression of *IL-6*, *IL-8*, *IP-10*, *VCAM-1*, *TNFR2* and *IL-1RII* mRNA and secretion of IL-6, IP-10, MCP-1, sVCAM-1 and GM-CSF.

Finally, another important part regarding GEnC interaction with their microenvironment was covered via studying the effect of conditioned media from cytokine (TNF-α, IL-1β, IFN- α, IFN-γ)/LPS-stimulated conditionally immortalised human podocytes and mesangial cells. However, no statistically significant changes were observed in ciGEnC pro-inflammatory gene expression.

The THP-1 macrophage/ciGEnC *in vitro* model was therefore the one providing the most robust and interesting data.

The mRNA expression and/or secretion of seven experimentally validated urinary biomarkers for LN (AGP, CP, TF, L-PGDS, MCP-1, VCAM-1, NGAL) was tested following cytokine/LPS treatments (**Chapter 3**) and JSLE/HC serum treatments (**Chapter 4**). The ciGEnCs, however, exhibited substantial basal expression/secretion of only MCP-1 and VCAM-1 and this was statistically significantly increased only following cytokine/LPS treatments but not serum treatments.

It was therefore obvious that the human GEnCs are probably not the main source of these potentially novel LN urinary biomarkers. These biomarkers, however, could have a profound effect on ciGEnCs in terms of pro-inflammatory activation. For this reason, the human ciGEnCs were treated for 24 hours with human recombinant biomarkers (apart from VCAM-1) alone or combined all together. The viability of the ciGEnCs and the expression and secretion of the pro-inflammatory genes/proteins previously tested on Chapters 3 and 4 was then studied. The urinary biomarkers, however, had no profound effect on ciGEnC viability or pro-inflammatory activation.

6.3. Important ciGEnC stimuli identified in this model of LN

The stimuli with the most profound effect on ciGEnC pro-inflammatory activation were the four cytokines (TNF-α, IL-1β, IL-13, IFN-γ), LPS, the THP-1 conditioned media and to a smaller extent the serum treatments (**Figure 6.1**).

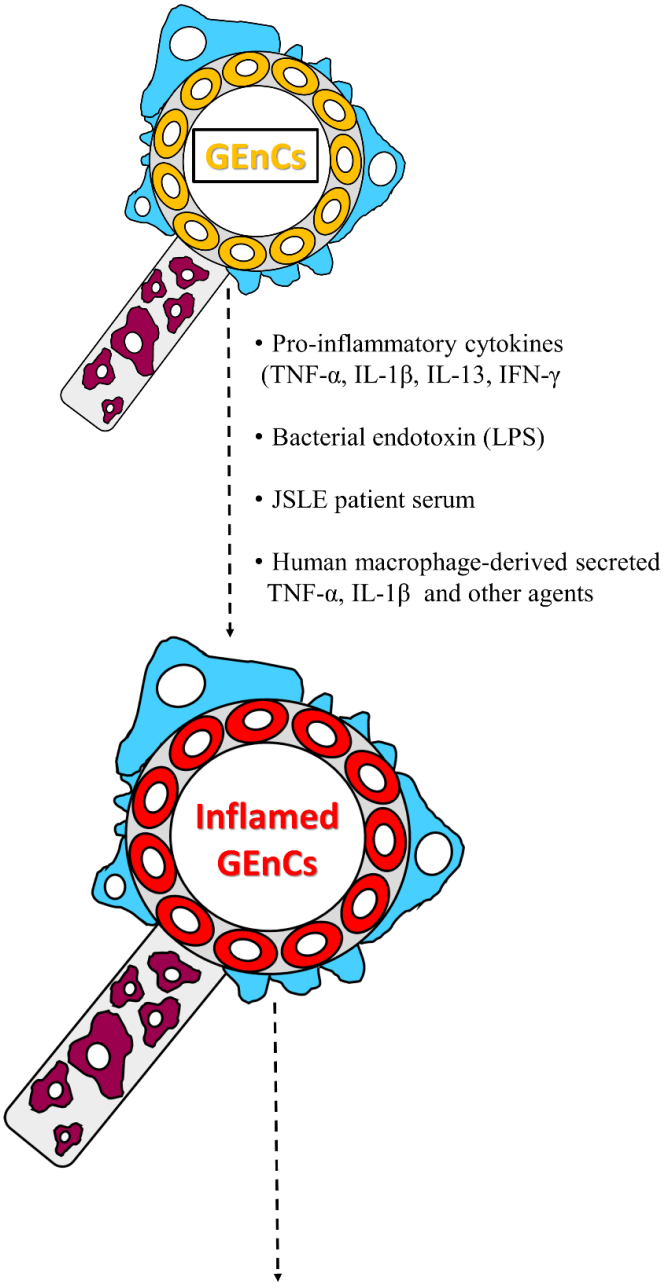


Figure 6.1 Important ciGEnC pro-inflammatory stimuli as identified in this study.

The most prominent effect of the JSLE serum treatments was the upregulation of *IL-6* mRNA expression which was mimicked by the ciGEnCs treatments with IL-1β and LPS; these led to statistically significantly increased secretion of IL-6. Furthermore, conditioned media from untreated THP-1 cells were able to significantly upregulate secretion of IL-6 compared to untreated ciGEnCs. When anti-TNF-α and IL-1Ra were added onto the THP-1 conditioned media, IL-6 levels were brought down to the levels of untreated ciGEnCs. This suggested that the presence of TNF-α and/or IL-1β within the JSLE sera could be playing a pivotal role in ciGEnC upregulation of IL-6 production.

The untreated THP-1 media also significantly increased IP-10 secretion compared to untreated ciGEnCs but anti-TNF-α and IL-1Ra addition only partially decreased the levels of IP-10, corroborating the finding that IFN-γ and LPS treatments had a robust effect on ciGEnCs IP-10 secretion, with TNF-α and IL-1β probably playing a more trivial role in IP-10 induction. The IFN-γ THP-1 media induced high levels of secreted IL-8 compared to the IFN-γ-treated ciGEnCs but although TNF-α and IL-1β treatments of ciGEnCs seemed to promote high levels of IL-8 secretion by the ciGEnCs, anti-TNF-α/IL-1Ra addition to the IFN-γ THP-1 media did not remarkably decrease IL-8 secretion. Therefore, other factors secreted by the IFN-γ-stimulated THP-1 cells could also promote IL-8 secretion (for example other members of the TNF superfamily or of the IL-1 superfamily).

Similarly, the IFN-γ THP-1 media promoted the secretion of MCP-1 by the ciGEnCs when compared to the IFN-γ-treated ciGEnCs and anti-TNF-α/IL-1Ra addition to the media considerably decreased MCP-1 levels, a finding agreeing with the TNF-α- and IL-1β-mediated increase of ciGEnCs MCP-1 secreted levels. sVCAM-1 secretion induced by the IFN-γ THP-1 media was significantly suppressed by anti-TNF-α/IL-1Ra addition to the THP-1 media, a result that supporting the finding that (along with IL-13),

TNF-α can promote VCAM-1 expression (and to a smaller extent sVCAM-1 secretion) by the ciGEnCs.

Finally, M-CSF secretion was significantly promoted by the untreated THP-1 media and GM-CSF secretion was upregulated by the untreated and IFN-γ THP-1 media. Anti-TNF-α/IL-1Ra addition to the THP-1 media led to decreased levels of GM-CSF, confirming the TNF-α- and IL-1β-mediated effect on elevated GM-CSF secretion by the ciGEnCs.

6.4. Clinical implications arising from these data

In this study, TNF-α and IL-1β have been identified as the most robust ciGEnC stimuli among the four cytokines found to have an effect on ciGEnC activation, leading to increased production of the majority of the pro-inflammatory proteins tested.

Anti-TNF-α antibody and IL-1Ra treatments had the desirable effect *in vitro* (inhibition of NF-κB nuclear translocation and decreased pro-inflammatory protein production), either when human recombinant TNF-α or IL-1β were added onto the ciGEnCs were directly inhibited or when more complex models were utilised to test their efficacy. Conditioned media from untreated, IFN-γ- and LPS-activated THP-1 macrophages were generated and the presence of TNF-α and IL-1β within these media was tested via ELISA. When anti-TNF-α antibody and IL-1Ra were added in these media, their pro-inflammatory effect on ciGEnCs was attenuated, providing evidence that the use of TNF-α and IL-1β blocking agents could be an efficient patient treatment in the future. However, to render human GEnCs into an intra-renal target for kidney-specific anti-TNF-α and IL-1Ra treatments, more future work is necessary including co-culture models with human podocytes and mesangial cells but also experimental animal models.

There are very many subsequent steps to take between an *in vitro* model that includes only one cell type and the development of a human GEnC-based therapeutic approach, especially given the mixed outcomes to date of anti-TNF-α usage in lupus patient clinics[444]. Heterozygous mice [NZB × B6, 129 Tnf^{0})F1] exhibiting decreased levels of TNF-α production were shown to develop autoimmunity and kidney disease with important disease features being aberrant upregulation of anti-nuclear autoantibody levels in the mice sera and abnormal expansion of anti-dsDNA-producing B cells[490]; these findings indicated an important role for TNF-α in the suppression of autoreactivity and suggested that lack of adequate TNF-α production could contribute to lupus development. On the contrary, on C3H.SW mice on which experimental SLE was induced via anti-DNA human autoantibody injection, early anti-TNF-α monoclonal antibody and pentoxiphylline administration suppressed TNF-α and IL-1β production and prevented experimental SLE development[491]. It has been suggested, that, TNF-α dysregulation and decreased production in both murine and human lupus is dependent on certain MHC alleles that have been connected to lupus-associated development of autoimmunity[492,493]. To this end, it is important to note that epistatic relationships between the TNF-α gene and major histocompatibility complex (MHC) genes can be occurring as, in both humans and mice, the TNF-α gene is located and is encoded within the MHC gene locus[494,495].

In humans, mixed results regarding TNF-α levels have also been observed in SLE patients[496,497]. In several studies, bioactive TNF-α levels within the sera of active SLE patients have been found to be remarkably increased and have been associated with lupus disease activity[498,499].On the other hand, another study has shown that serum TNF-α levels as well as the TNF-α/IL-10 ratio were upregulated in inactive SLE patients

compared to active SLE patients and healthy control, indicating a protective role of TNF-α in SLE patients[497].

In a study where both healthy and SLE blood donors serologically typed and genotyped for certain human leukocyte antigen (HLA) alleles (HLA-DR2, HLA-DQwl, HLA-DR3, HLA-DR4) were used to evaluate TNF-α production by peripheral blood lymphocytes[500], some very interesting results were generated. The HLA-DR2- and HLA-DQwl-positive healthy donors, often demonstrated low TNF-α levels in contrast to HLA-DR3- and HLA-DR4-positive healthy donors who exhibited high TNF-α production levels[500]. Similar to healthy subject, in HLA-DR2- and HLA-DQwl-positive SLE patients low TNF-α levels were observed and these alleles were linked to more frequent occurrence of LN[500]. In contrast to HLA-DR2- and HLA-DQwl-positive SLE patients and similar to the healthy individuals, the HLA-DR3-positive patients exhibited induction of high TNF-α levels and no LN predisposition, whereas the HLA-DR4-positive patients demonstrated high TNF-α levels with the latter negatively correlating with LN development[500]. It is therefore suggested by these findings that LN predisposition could be linked to low TNF-α production and that SLE is not a disease with a homogeneous pattern of pathogenic features. On the contrary, it is suggested that patients could be divided into two different categories that would consequently determine the success of an anti-TNF-α treatment; those who are HLA-DR2/DQwl-positive, produce low TNF-α levels and are genetically predisposed to LN and those who are HLA-DR3/DR4-positive, produce high TNF-α levels and have no genetic predisposition to LN development.

It would therefore be interesting if future laboratory studies on ciGEnCs could also focus on further elucidating any potential relationships between production of TNF-α and of MHC molecules following pro-inflammatory stimulation. Furthermore, plasma

and serum studies of JSLE patients stratified into active LN and inactive LN patients with the latter being further subdivided into those with previous renal involvement and those with no signs of renal disease could focus onto exploring potential correlations of plasma/serum TNF-α levels with the presence of specific HLA alleles (HLA-DR2, HLA-DQwl, HLA-DR3, HLA-DR4).

In both SLE and JSLE, IL-1β patient polymorphisms in the promoter region of *IL-1β* gene rendering IL-1β overactive have been discovered[355,501], and therefore this finding could open new avenues for therapeutic approaches. Similarly, patient polymorphisms in the promoter region of the *TNF-α* gene being linked to LN predisposition have also been discovered[502,503].

Infliximab (a chimeric antibody) and Etanercept (a fusion protein), both TNF-α blockers, have been used in rheumatic diseases including rheumatoid arthritis (RA), psoriasis, Crohn's disease, juvenile idiopathic arthritis, but also SLE. However, the TNF-α blockers did not only have successful results but have been known to induce autoantibody production and lupus-like syndrome in patients suffering from other autoimmune diseases than SLE[504–506]. In an open-label study, SLE patients (with either LN or arthritis) refractory to other therapies were treated with infliximab in combination with azathioprine or methotrexate and demonstrated decreased disease activity-the nephritic patients showed attenuation of proteinuria and the arthritic patients showed remission of arthritis- although some developed urinary tract infections[507]. A long-term follow-up study by the same group, however, showed that long-term infliximab therapy -in contrast to short-term therapy- led to serious and severe adverse effects in patients, even cerebral lymphoma[165]. Anakinra, a non-glycosylated form of human IL-1Ra, has been widely used in systemic juvenile-onset RA patients[445], was also able to improve arthritis symptoms in SLE patients with safety and efficacy[508].

This ciGEnC study mainly focused on testing the effect of anti-TNF-α/IL-1Ra treatments as TNF-α and IL-1β treatments promoted the majority of pro-inflammatory proteins tested. Nonetheless, IFN-γ was found to significantly promote IP-10 and IL-10 secretion -with IFN-α potentially contributing in even higher IP-10 and IL-10 levels as suggested by the combined cytokine treatments- whereas IL-13 had a specific effect on VCAM-1 production. It would therefore be very interesting but also important in terms of potential clinical implications, that future *in vitro* studies test the effect of IFN-γ, IFN-α and IL-13 blockade/neutralisation in ciGEnC production pro-inflammatory proteins.

In SLE patients, dose-response intravenous treatments with sifalimumab or rontalizumab, anti-IFN-α monoclonal antibodies, induced a dose-depedent reversal of the blood and skin type I interferon signature[509]. Furthermore AMG 811, an anti-IFN-γ monoclonal antibody, has been used in Phase Ib clinical trial in SLE patients with or without LN with good safety results, although further investigation is required to determine the usefulness of this agent in clinic[510]. In SLE or JSLE, no anti-IL-13 monoclonal antibody or any other anti-IL-13 treatment has been used yet. It would however be interesting if anti-IL-13 antibody treatments were used *in vitro* to test their effect on human ciGEnCs and similarly, future studies could also investigate the effect of anti-IFN-α and anti-IFN-γ antibody treatments on ciGEnCs, especially after ciGEnCs treatments with pro-inflammatory stimuli-activated conditioned media derived from human immune cells, such as macrophages and T cells.

6.5. STRING analysis for the urinary biomarkers

In this study, the novel, laboratory-tested human urinary biomarkers for LN (AGP or ORM1, L-PGDS or PTGDS, TF, CP, NGAL or LCN2, MCP-1 or CCL2, VCAM-1) were not found to have any remarkable effect either on ciGEnCs' pro-

inflammatory phenotype or on ciGEnCs' viability. Furthermore, the ciGEnCs were found to substantially express and produce only MCP-1 and VCAM-1.

For these reasons, and to explore future avenues for *in vitro* laboratory research involving these biomarkers, a STRING network analysis was performed[511] to investigate potential interactions among biomarkers and interesting pathways into which they could be involved. The protein-protein interaction (PPI) p-value was 2.14×10^{-7}, indicating a strong possibility that real protein-protein interactions exist among the seven biomarkers (**Figure 6.2**).

Associations traced from curated databases (light blue line), co-expression relationships (black line), textmining-derived (co-mentioned in PubMed abstracts) interactions (light green line) and experimentally determined interactions (fuchsia line) were found among the biomarkers (**Figure 6.2**). AGP (or ORM1), NGAL (or LCN2), MCP-1 (or CCL2) and VCAM-1 were shown to be involved in immune system-related pathways (node colour: green) in contrast to TF, CP and L-PGDS (or PTGDS) that might be mainly involved in metabolism-related pathways (**Figure 6.2**).

VCAM-1 and MCP-1, the two biomarkers substantially produced by ciGEnCs and remarkably modified by pro-inflammatory stimuli, were found to be involved in fluid shear stress-related pathways in atherosclerosis (node colour: red) (**Figure 6.2**). Having previously mentioned that fluid shear stress is an important player involved in GEnC pro-inflammatory activation[30] that requires future investigation, it would be interesting to *in vitro* imitate fluid shear stress conditions; subsequently, potential changes in levels of MCP-1 and sVCAM-1 secretion as well as surface VCAM-1 expression on ciGEnCs could be tested. Similarly, the effect of IL-17 on ciGEnCs, was not tested in this study and, yet, IL-17 is a cytokine with an important role in lupus as it

is involved in aberrant T cell responses[512]. In this analysis, MCP-1 (or CCL2) and NGAL (or LCN2) were shown to be involved in the IL-17 signaling pathway (node colour: purple) (**Figure 6.2**) and thus future *in vitro* studies could explore changes in MCP-1 ciGEnC production following ciGEnC stimulation with IL-17 directly or, potentially, with T cell condtioned media (in which IL-17 presence will have been previously determined). NGAL was not found to be expressed by the ciGEnCs but it would be interesting to test the possibility of IL-17 specifically inducing NGAL expression by the ciGEnCs.

Finally, MCP-1 and VCAM-1 were found to be involved in the pathway of advanced glycation end products (AGEs) and their receptor (RAGE) in diabetic complications (node colour: fuchsia) (**Figure 6.2**). More specifically, the AGE/RAGE signaling system has been found to to be involved in diabetic nephropathy via inducing vasculopathy; AGEs can elicit their effect via interaction with glomerular endothelium-expressed RAGE, leading to endothelial cell oxidative stress and inflammation and activation of pathways such as the NF-κB pathway[513–515]. Therefore, future work could explore the effect of AGE treatments on ciGEnC pro-inflammatory activation whereas JSLE patient -with or without LN- plasma and serum samples could be tested for the presences of AGEs and/or soluble RAGE. Furthermore, ciGEnC treatment with LN-related cytokines (such as TNF-α and IL-17) could give interesting results regarding ciGEnC surface expression of RAGE.

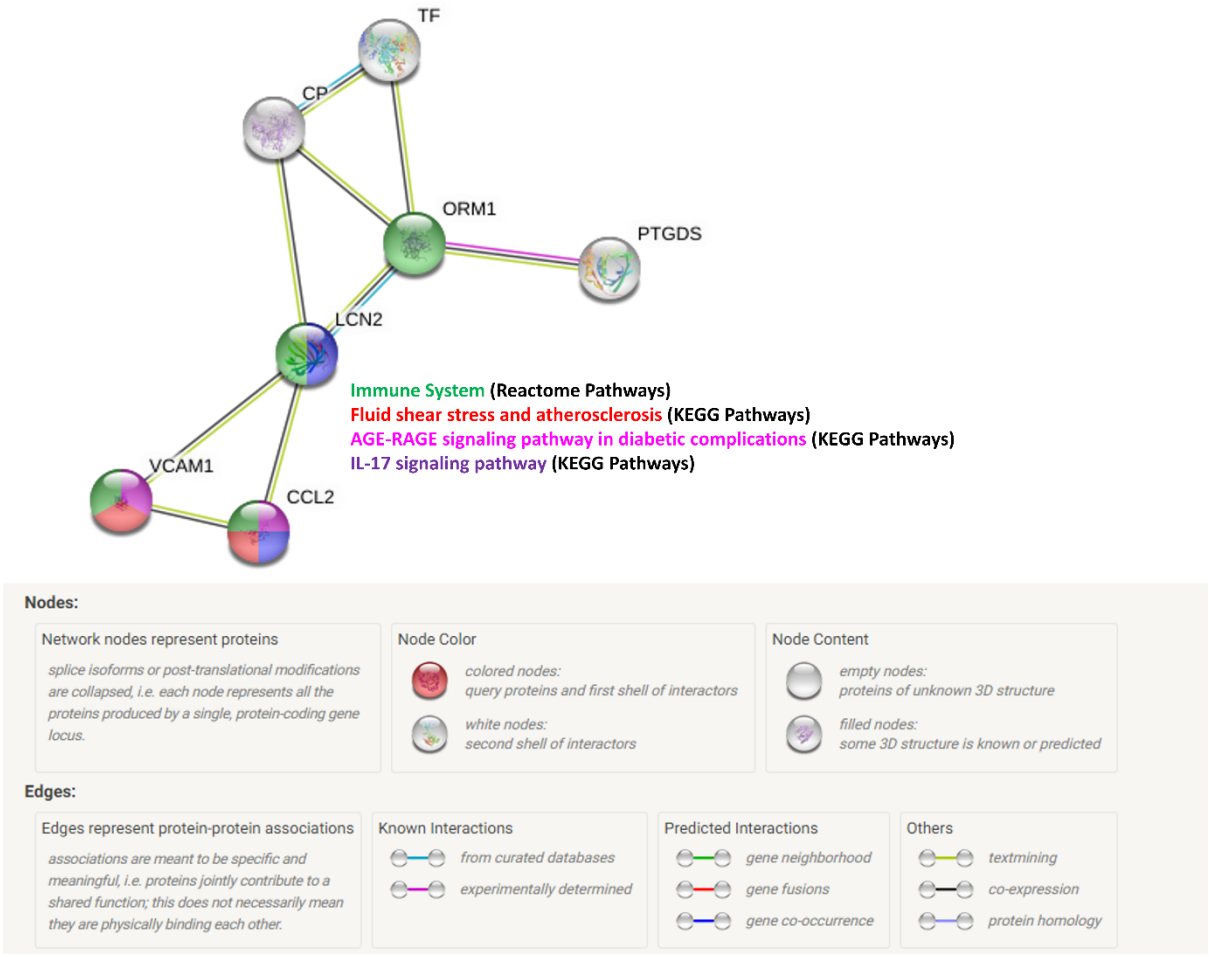


Figure 6.2 STRING analysis for the novel urinary biomarkers (ORM1=AGP, PTGDS=L-PGDS, LCN2=NGAL, CCL2=MCP-1).

6.6. Study limitations and the importance of specifically studying the human GEnCs

As every *in vitro* study, this study had several limitations. First of all, an *in vitro* model cannot precisely imitate the procedures occurring within a live human organism or the complexity of the human renal structure. Also, only single ciGEnC culture was performed and no 2-D or 3-D co-culture models with ciGEnCs-podocytes-mesangial cells were developed; these models could be more accurate as cell-cell interactions affect and modulate the actions of a cell type along with factors secreted within their

microenvironment. Therefore, no cell-cell contact effect either between ciGEnCs and ci podocytes/mesangial cells or between ciGEnCs and neutrophils/macrophages was studied, which means that only the paracrine effects mediated by secreted factors were investigated. Fluid shear stress is another extremely important factor that could affect the behaviour of the human GEnCs along with pro-inflammatory stimulation and thus, future *in vitro* models using ciGEnCs should also investigate this parameter.

With the serum samples, no concentration response was performed due to the limited number of samples available from active disease patients and this, together with the medications administered to patients, limits the possibilities of observing a strong, robust effect mediated by the serum samples. The time-points used in the study were also limited at 24 hours and in some cases also 4-hour time-points were used. Time response experiments expanding on more acute (1 hour or 2 hours) and more chronic time-points (48 hours-72 hours-96 hours-7 days) should definitely be performed in the future as LN is a chronic inflammatory disease.

In a study in which 76 SLE patients (85% were women and 67% had biopsy-proven LN) were enrolled along with 42 disease controls (having other forms of renal disease) and 80 HCs, the serum levels of IgA- anti-endothelial cell antibodies (AECA) were evaluated[516]. AECA had been previously found to cause HUVEC and human kidney endothelial cell dysfunction[517,518] thus, this study also investigated their effect on HUVECs and primary human GEnCs[516]. The IgA-AECA prevalence in the sera of SLE patients with and without LN was 35% and 28% respectively whereas in the disease controls and HCs the IgA-AECA prevalence was 2% and 7% repsectively[516]. Furthermore, IgA-AECA levels correlated with disease activity and active renal lesions[516]. The human GEnCs were incubated for 24 hours with 5% sera from AECA-positive LN patients and their proliferation rates that were measured via confluence

assay at both 0 and 24 hours indicated that the IgA-AECA sera could significantly decrease the GEnCs' proliferation rates[516]. This finding suggests that the IgA-AECA can recognise and potentially bind to the human GEnCs and induce GEnC damage.

It is important to note that the 24-hour use of 5% sera concentration, same time-point and same serum concentration used for this PhD research project, induced significant reduction in the ability of primary human GEnCs to proliferate. The 24-hour serum effect on ciGEnC proliferation, however, was not tested in this study and thus, it would be very interesting if future studies could investigate a potential LN serum effect on ciGEnC proliferation but also ciGEnC death.

In a recent *in vitro* study using SLE sera from 25 patients and HC sera from 14 healthy individuals, HUVECs were *in vitro* treated with 20% sera (for 6 or 24 hours) to detect changes in endothelial NO synthase (eNOS) expression and with 50% sera to test changes in HUVEC NO production and in neutrophil migration and adhesion[519]. The SLE sera were able to significantly induce increased eNOS mRNA expression but at the same time to reduce NO production[519]. Furthermore, the SLE sera significantly promoted neutrophil migration towards the HUVECs[519]. With NO production being an important endothelial cell function, it would be interesting if future ciGEnC treatments with LN sera were performed to assess changes in NO production and eNOS expression.

In a study testing the effect of uremic damage on human endothelial cells pooled human serum from HCs and patients with CKD suffering from uremia was used to treat HUVECs[520]. More specifically, HUVECs were treated with four different groups of pooled human sera including, HC sera, pre-dialysis patient sera, peritoneal dialysis patient sera and haemodialysis patient sera[520]. The cells were stimulated for 1, 5 and 15 minutes with 2% human pooled sera from the four different groups and subsequently,

activation of NF-κB and of the p38 mitogen-activated kinase pathway (MAPK) was assessed[520]. The sera from the three groups of CKD/uremic patients were able to significantly promote activation p38 MAPK and NF-κB compared to the HC sera at 1 and 15 minutes[520]. Similar to the HUVECs, 10% sera from LN patients have been shown to significantly enhance neutrophil chemotaxis toward primary human GEnCs compared to 10% HC sera[521].

The most notable effect in the activation of these pro-inflammatory pathways was attributed to the pooled sera from the peritoneal dialysis patients a finding that correlated with increased presence of endothelial damage markers -soluble VCAM-1, ICAM-1, vWF, E-/P-selectin and elevated levels of circulating endothelial cells-, especially in the plasma of the peritoneal dialysis patients[520]. It is important to note here that use of only 2% serum concentration was able to induce an important effect in the activation of important pro-inflammatory pathways in HUVECs *in vitro*, suggesting that the use of pooled sera could have a robust effect in endothelial cell activation. Thus, the use of pooled active and inactive LN sera to treat ciGEnCs *in vitro*, could be considered in the future. Serum starvation prior to treatments with HC and patient sera should also be considered.

When human GEnCs, labeled for detection of glycosaminoglycans (GAGs), were exposed for 24 hours in 10% plasma from patients with heavy albuminuria or 10% plasma from HCs, the GAGs, which are glycocalyx components, were reduced following albuminuria patient plasma activation[522]. The endothelial glycocalyx is a very important part of the human GEnCs that was not studied in this project. It would therefore be important to elucidate the role and modulation of the endothelial glycocalyx composition in lupus renal disease with the *in vitro* use of human ciGEnCs.

When human arterial and human venous endothelial cells were treated for 24 hours with 20% sera collected from end-stage renal failure patients before and after hemodialysis treatment, interesting changes occrurred[523]. The post-hemodialysis patient sera induced significant changes in both arterial and vein endothelial cells compared to the pre-hemodialysis sera, but in a completely opposite way[523]. More specifically, the post-hemodialysis patient sera were able to increase the secretion -among others- of IL-6 and vWF by venous endothelial cells whilst in the same time they significantly reduced IL-6 and VEGF synthesis by the arterial endothelial cells[523].

In another study in which HUVECs were *in vitro* treated for different time-points (8-72 hours, depending on the experimental assays) with 5%, 10% and 20% pre- and post-hemodialysis sera from patients with CKD the effect of hemodialysis treatment, a process that leads to removal of uremic toxins (such as urea) from the CKD patients, was highlighted[524]. In contrast to the post-hemodialysis serum treatments, the pre-hemodialysis serum treatments induced higher apoptotic HUVEC levels, reduced the cell numbers, and decreased the number of migrating endothelial cells in a wound-healing assay, in a dose-dependent manner[524]. Furthermore, the pre-hemodialysis 20% sera were able to significantly increase the production of ECM-degrading and ECM-remodeling proteins, such as TIMP1, TIMP2, collagen IV and elastin[524]. In this research project, *TIMP1* and *COL1A1*, *COL1A2*, *COL3A1* and *COL4A1* mRNA expression were tested, but without revealing intriguing changes following treatments with active LN, inactive LN and HC sera. Future work could further investigate the serum effect on *TIMP2* and elastin mRNA expression as well as on the expression of more types of collagen.

It also has to be noted that not all cytokines involved in JSLE and LN were tested; for example, the effect of IL-17A on ciGEnC activation was not investigated. IL-17A

has been shown to be produced by kidney-infiltrating T cells and promotes local renal inflammation in lupus disease, via various mechanisms including cytokine and chemokine production and immune cell infiltration[512,525]. Similar to IL-17A, the effect of adaptive immune cells and more specifically the effect of T cells on ciGEnCs and chronic renal disease was not investigated, although Th17 cells have been observed to promote kidney injury in LN[526]. Instead, only the effect of innate immune cells (neutrophils and macrophages) was investigated in this study. Finally, only immune cells derived from healthy adult donors and not from JSLE patients were utilised in this study.

The novelty of data and findings arising from this work lies in the fact that the human endothelial cells are not a homogeneous cell population located in a specific part of the human body. On the contrary, many different types of human endothelial cells can be found in a plethora of tissue microenvironments located in organs such as the heart, brain, eye, lungs, skin, kidney, the placenta and other organs. The endothelial cells line the lumen of veins and arteries as well as of larger or smaller blood vessels and capillaries.

Thus, although the different types of human endothelial cells share a number of signature functions (blood circulation-tissue barrier, angiogenesis, blood flow homeostasis and tight regulation of vasodilation/vasoconstriction, response to pro-inflammatory stimuli and to oxidative stress, fluid shear stress sensing), they can also attain relatively altered phenotypes that accommodate the specific requirements of the local microenvironment at which they are present. This could mean that depending on the origin of each vascular bed, the endothelial cells could exert their commonly shared basic functions via exhibiting differential responses to certain stimuli and via using differential mechanisms and/or effector molecules.

For example, vWF, an important endothelial cell-specific marker involved in platelet adhesion and blood co-agulation[527], has been shown to be more highly expressed in veins compared to arteries and arterioles[528]. Such differences, have been suggested to occur under the influence of certain local microenvironments, such as the cardiac mircoenviromnment[528]. In a study in which endothelial cells were isolated from four main human foetal organs –kidneys, liver, heart and lung- vWF protein expression was tested[529]. The heart endothelial cells displayed high vWF expression and the lung endothelial cells exhibited in general high vWF levels, comparable to those expressed by the heart endothelial cells[529]. In the liver, the endothelial cells expressed lower vWF levels[529]. Within the kidney, the GEnCs expressed higher vWF levels compared to the peritubular microvascular endothelial cells[529]. Differences in the structure of fenestrations among endothelial cells in different organs were also observed[529].

Atherosclerosis leading to vascular lipid deposition and chronic inflammation, principally affects the arteries –including the aorta- and not the veins[530]. For this reason, an *in vitro* study comparing the expression profiles of atherosclerosis-related genes in primary human saphenous vein endothelial cells (HSVECs) versus primary human coronary artery endothelial cells (HCAECs) was performed[530]. This study revealed striking differences between the HSVECs and HCAECs at both unstimulated and oxidative stimuli-induced conditions; in the HSVECs, atheroprotective genes involved in cell growth (important for the integrity of the endothelium), oxidoreductase activity, extracellular matrix deposition and anti-inflammatory responses were found to be significantly upregulated and more highly expressed compared to the coronary artery endothelial cells[530]. Furthermore, genes involved in the NF-κB pathway were found to be downregulated by the HSVECs[530]. In contrast, genes involved in adhesion, apoptosis

and cellular proliferation, functions tightly-associated to atherosclerosis, were found to be significantly upregulated by the HCAECs[530].

In another *in vitro* study, neutrophil recruitment following TNF-α and IL-1β stimulation under static and blood flow (via shear stress application) conditions was compared among HUVECs, human umbilical arterial endothelial cells (HUAECs) and human coronary arterial endothelial cells (HCAECs)[531]. Although under static conditions all three types of TNF-α and IL-1β-stimulated endothelial cells were able to effectively recruit neutrophils, under shear stress conditions TNF-α and IL-1β-stimulated HUAECs and HCAECs lost their ability to recruit flowing neutrophils[531]. On the other hand, HUVECs were still able to efficiently recruit neutrophils after IL-1β but not after TNF-α stimulation, an effect that could largely be attributed not only to the shear stress force but also to the lack of human recombinant basic FGF (that is able to bind to the basement membrane) within the HUVEC media (in contrast, FGF was present in both HUAEC and HCAEC media)[531]. Another study using HUVECs has shown that under prolonged culture of HUVECs, basement membrane deposition could impair TNF-α-mediated neutrophil recruitment[532]. Furthermore, in bovine aortic endothelial cells changes in the subendothelial ECM composition have been shown to influence shear stress-induced NF-κB activation[533]. Thus, it seems that under local shear stress and changes in basement membrane and ECM composition, the endothelial cells can differentially alter their phenotypes.

The importance of vascular bed-specific flow patterns has also been stressed by another *in vitro* study, using HSVECs and HCAECs[534]. HSVECs and HCAECs were stimulated with TNF-α and were both exposed to either coronary artery flow or venous artery flow; the coronary artery flow was physiological for the HCAECs and the venous artery flow was physiological for the HSVECs[534]. Thus, when the TNF-α-stimulated

HSVECs were exposed to coronary artery flow, mononuclear cell adhesion was increased compared to both TNF-α-stimulated HSVECs and HCAECs exposed to coronary artery flow[534]. This increased mononuclear adhesion was accompanied by increased HSVEC secretion of soluble VCAM-1, ICAM-1 and E-selectin compared to HCAECs[534]. In terms of adhesion molecule mRNA expression, only coronary artery flow exposure could induce HSVEC *VCAM-1* mRNA expression along with *ICAM-1* and *E-selectin* mRNA expression whereas venous artery flow induced only *ICAM-1* and *E-selectin* mRNA expression[534]. Venous and artery flow exposure had a strikingly different effect on TNF-α-stimulated HCAECs (reduced *VCAM-1* and *E-selectin* mRNA expression) but coronary artery flow was able to upregulate HCAEC *ICAM-1* mRNA expression[534].

In the same study that assessed vWF expression in human endothelial cells isolated from four principal organs –heart, lung, kidney, liver-[529], RNA-sequencing was performed using *in vitro* cultured human heart, lung, kidney and liver endothelial cells, to detect potential organ-specific gene expression signatures. All four types of human endothelial cells demonstrated a common and high expression of transcription factors (and co-factors) involved in vascular development and remodelling as well as in VEGF signaling[529]. At the same time, all four types of human endothelial cells are also differentially expressing genes involved in organ-specific growth and development with the kidney endothelial cells expressing higher levels of genes involved in regionalisation, pattern specification processes, nephron development, embryonic organ morphogenesis and hormone secretion and transport (*Angpt2*, *HOXB7*, *HOXD8*, *HOXA9, HOXB9, EXOC3L2, ZNF366, ZNF503-AS1, ELAVL2, NLGN4X, MMP7*)[529]. The homeobox (HOX) transcription factor genes that were particularly enriched by the human kidney endothelial cells are highly involved in kidney development and

patterning[535,536] and thus, modulations in their expression levels/epigenetic pattern under inflammatory conditions could be studied in ciGEnCs in the future.

In this PhD research project, two important adhesion molecules were found to be upregulated by the ciGEnCs following pro-inflammatory stimulation; VCAM-1 and ICAM-1. The cytokines and LPS tested, had a differential effect on ciGEnC surface expression of VCAM-1 and ICAM-1, suggesting that different pro-inflammatory stimuli could lead to recruitment of different kidney-infiltrating immune cell populations. IL-13 had an effect exclusively on surface VCAM-1 expression, only in combination with TNF-α. ICAM-1 expression was significantly increased by TNF-α, IL-1β and LPS. 24-hour ICAM-1 upregulation by TNF-α, IL-1β and LPS has also been confirmed in HUVECs[385,537]. Similar to the human ciGEnCs and in contrast to other types of human endothelial cells (carotid, coronary endothelial cells), lack of 24-hour IL-1β- and LPS-mediated upregulation of surface VCAM-1 has been previously observed in HUVECs and human subclavian endothelial cells[537].

Thus, it is important, to highlight that this project is further enhancing the notion supported by a variety of previous studies that the local microenvironment into which the human endothelial cells are found, can significantly influence and alter their response to a range of stimuli. The human endothelial cells are therefore generally characterised by high heterogeneity and by unique phenotypes and no definite conclusions should ever be extrapolated from general results of studies using human endothelial cells different than those of specific interest. Hence, further human GEnC-specific studies are necessary to elucidate and accurately depict their unique phenotype.

6.7. Future work: Taking human GEnC research to the next step

This study was mainly focused on investigating the pro-inflammatory profile of human GEnCs in the context of LN. However, the human GEnCs have more than one important function.

Apart from being potent pro-inflammatory mediators of renal pathogenesis, they are also an integral part of the GFB and via their glycocalyx and fenestrations they can regulate both the GFB net charge and permeabilty signficantly contributing to the highly selective filtering capacity of the GFB. It is therefore very possible that these important functions of ciGEnCs could be significantly impaired upon renal inflammation. For this reason, studying the effect of pro-inflammatory activation on ciGEnC permeability *in vitro* (for example via albumin permeability transwell assays) would be a very important step onto elucidating all the different procedures and mechanisms of GEnC involvement in renal disease. Cytoskeleton remodelling and changes in fenestration number or structure could also generate interesting findings in terms of human GEnC research.

Furthermore, it is necessary that more chronic models of ciGEnC stimulation should be introduced in order to be able to identify potential structural and functional changes during the course of the renal disase. To this end, co-culture models with podocytes, mesangial cells and both innate and adaptive immune cells derived from JSLE patients would be of paramount importance in order to create more realistic *in vitro* models and thus, provide more meaningful and robust findings.

6.8. Final Conclusions

This study aimed to address the hypothesis that the human GEnCs are not just passive bystanders but are actively involved in the development and perpetuation of renal inflammation in LN.

This study has demonstrated that:

1. The human ciGEnCs respond to pro-inflammatory cytokines (TNF-α, IL-1β, IL-13, IFN-γ) and LPS via significantly upregulating production of various pro-inflammatory proteins and via inducing NF-κB and STAT-1 activation, while retaining their viability.

2. The human ciGEnCs can be affected by their surrounding microenvironment to express pro-inflammatory and pro-fibrotic genes, as shown by serum and THP-1 macrophage conditioned media treatments.

3. No ciGEnC pro-inflammatory phenotype induction or apoptotic/necrotic cell death occurred following treatments with novel laboratory LN urinary biomarkers.

Whilst acknowledging the limitations of this study, important results were generated, demonstrating that human GEnCs are in fact potent producers of pro-inflammatory cytokines, chemokines, blood cell growth factors, adhesion and co-stimulatory molecules. In particular, these are all important factors that have been associated with the aetiopathogenesis of LN and/or JSLE itself (**Table 6.1 & Figure 6.1**).

The human GEnCs could therefore be a promising target for kidney-specific therapies in LN.

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APPENDIX A: RESEARCH ETHICS APPROVAL

A1: JSLE patient and paediatric control sample ethics

NHS
Health Research Authority

North West - Liverpool East Research Ethics Committee

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09 February 2016

Professor Michael W. Beresford
Professor in Child Health
University of Liverpool
Institute of Child Health
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Dear Professor Beresford

Study title:	**UK Juvenile Systemic Lupus Erythematosus Cohort Study & Repository: "Clinical characteristics and immunopathology of juvenile-onset systemic lupus erythematosus"**
REC reference:	**06/Q1502/77**
EudraCT number:	**N/A**
Amendment number:	**Substantial Amendment 4**
Amendment date:	**01 January 2016**
IRAS project ID:	

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

Approval was sought for updates made to the protocol.

The Sub-Committee commented that page 11 of the protocol stated that you are proposing to include healthy controls, aged 13-15 from friends and siblings and they requested further information on how these controls would be approached, who will approach them, etc.

You replied that you have provided more detailed information on how you plan to approach these healthy controls in the protocol on page 11 with the following text:

"Visitors aged 13 – 15 years attending the hospital may also be approached where appropriate for example through asking patients to identify friends/siblings who may be willing to take part in the study. Visitors will only be approached in an appropriate, confidential, quiet environment by GCP trained staff on the delegation log. Staff will only approach potential participants where they feel comfortable that it is appropriate timing and the family may be interested. They will provide an appropriate patient information leaflet and offer time to take the information leaflet away and read it in their own time. They will be clear that the family may say no. They will typically be approached by a doctor or nurse during a

clinic setting when the family are attending for another reason, for example, asking patients / parents if they have siblings / other children who may be interested in taking part in the study."

The Sub-Committee also sought further information on where the adult controls would come from, how and who will be approaching them.

You replied that more detailed information on how you plan to approach adult healthy controls in the protocol on page 11 with the following text:

"Adults aged sixteen years or over, without any significant past medical history of inflammatory disease and freely able to consent will be recruited to assist with experiment optimisation at AHCH. Persons working in the Trust or the University (e.g. medical students / administration staff etc.), will be approached and provided with an information sheet. Staff will be approached by GCP trained staff who are familiar with the study and on the study delegation log. They will only be approached in an appropriate, confidential and quiet environment and provided with an information sheet. They will be offered time to take the information leaflet away and read it in their own time. Staff will be clear that they can say no and there is no requirement for them to take part in the study. Only staff that are willing to take part in the study will be asked to sign the consent form."

The Sub-Committee commented that on page 14 of the protocol it stated that the only identifiable details to be stored electronically are email address; the Sub-Committee sought further clarification as to why you can't store these separately and just access them when required.

You replied that you agree that email addresses can be stored separately and accessed when required. You have removed this from the protocol on page 14.

The Sub-Committee asked for clarification on how much blood is to be taken, for example, will it be 5ml/1 teaspoon. They requested that the PILs are changed to state the amount of blood to be taken.

You replied that the volume of blood to be taken is 2-3 teaspoons. You have revised the PILs and resubmitted them.

The Sub-Committee requested that in the section *"Do I have to help"* in the PILs, it should start with "no". They noted that some of the PILs already do this, but not all of them.

You updated all the PILs and resubmitted.

In the PILs for the inflammatory controls for <6 and 6-12, the Sub-Committee noted that it does not state that participants have been chosen because they have arthritis, but does in the PILs for 13-15 year olds and sought further clarification as to why this is the case.

You updated the PILs accordingly and resubmitted.

The Sub-Committee commented that the PILs do not mention being flagged with the HSCIC, or informing the GP and sought further clarification for this.

You replied that you are only using HSCIC flagging on JSLE recruits and this was already included on the PILs for the 13-15 year olds, >16 year olds and parents. You said that you would not be using the HSCIC service for any of the control recruits. You also said that informing the GP was already included in all the PILs except for the adult healthy controls, which you amended and resubmitted.

The Sub-Committee commented that there was no contact information provided for complaints in the PILs.

You replied that you have updated the PILs to include the following in the parent and participant >16 years PILs:

"What do we do if there is a problem?
If you have a problem you can speak to any member of the study team and we will try to help. If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team."

The Sub-Committee sought further clarification as to whether participants can still take part if they do not agree to flagging with the HSCIC and/or if they do not want DNA analysis.

You replied that patients can still be involved in the study if they don't agree to flagging with the HSCIC and/or if they don't want DNA analysis by not initialling the appropriate boxes on the consent form. Each line of the consent form needs to be initialled specifically, and any not included such as these are noted. You said that to date, none have not wanted to take part in all of the components, but it remains always an option. You also mentioned that this is already included in the protocol for HSCIC on page 16.

You went to say that you have updated the protocol regarding DNA analysis on page 18 with the following sentence:

"Participation in this section of the study requires the patient to specifically initial a box saying 'I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else'. Patients who do not want their DNA to be analysed can still participate in the rest of the study."

You went on further to state that you have included an additional point on the assent/consent forms regarding DNA analysis:

"I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else."

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		29 December 2015
Notice of Substantial Amendment (non-CTIMP)	Substantial Amendment 4	01 January 2016
Other [Response to Committee Queries]		05 February 2016
Participant consent form	4	01 January 2016
Participant consent form [Controls]	4	01 January 2016

Participant information sheet (PIS) [Controls]	4	01 January 2016
Participant information sheet (PIS)	4	01 January 2016
Research protocol or project proposal	4	01 January 2016

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

06/Q1502/77:	**Please quote this number on all correspondence**

Yours sincerely

On behalf of
Mrs Glenys J Hunt
Chair

E-mail: nrescommittee.northwest-liverpooleast@nhs.net

Enclosures: List of names and professions of members who took part in the review

North West - Liverpool East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Glenys J Hunt Chair	Solicitor	Yes	
Dr Peter Walton	Lay Member	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Ewa Grzegorska	REC Assistant

A2: Adult volunteer donor ethics

From: Ethics
Sent: 26 January 2015 17:10
To: Flanagan, Brian
Subject: RE: RETH000773: request for review of study ethcs application.

Dear Dr Flanagan,

I am pleased to inform you that the Sub-Committee has approved your application for ethical approval for your study. Details and conditions of the approval can be found below.

Reference:	RETH000773
Sub-Committee:	Physical Interventions
Review type:	Full committee review
Principle Investigator:	Dr Brian Flanagan
Department:	Women's and Children's Health
Title:	Analysis of normal blood leukocyte function using cells from healthy volunteers.
First Reviewer:	Professor Graham Kemp
Date of initial review:	11/12/2014
Date of Approval:	26/01/2015

The application was APPROVED subject to the following conditions:

Conditions

All serious adverse events must be reported to the Sub-Committee within 24 hours of their occurrence, via the Research Integrity and Governance Officer (ethics@liv.ac.uk).

This approval applies for the duration of the research. If it is proposed to extend the duration of the study as specified in the application form, the Sub-Committee should be notified. If it is proposed to make an amendment to the research, you should notify the Sub-Committee by following the Notice of Amendment procedure outlined at http://www.liv.ac.uk/media/livacuk/researchethics/notice%20of%20amendment.doc. If the named PI / Supervisor leaves the employment of the University during the course of this approval, the approval will lapse. Therefore please contact the Research Integrity and Governance Officer at ethics@liverpool.ac.uk in order to notify them of a change in PI / Supervisor.

Kind regards

Matthew Billington
Research Integrity and Governance Officer

Research Support Office
University of Liverpool
Waterhouse Building (2nd Floor, Block C)
3 Brownlow Street
Liverpool
L69 3GL

Email: ethics@liverpool.ac.uk
Telephone: 0151 794 8290
Website: Research Integrity & Ethics

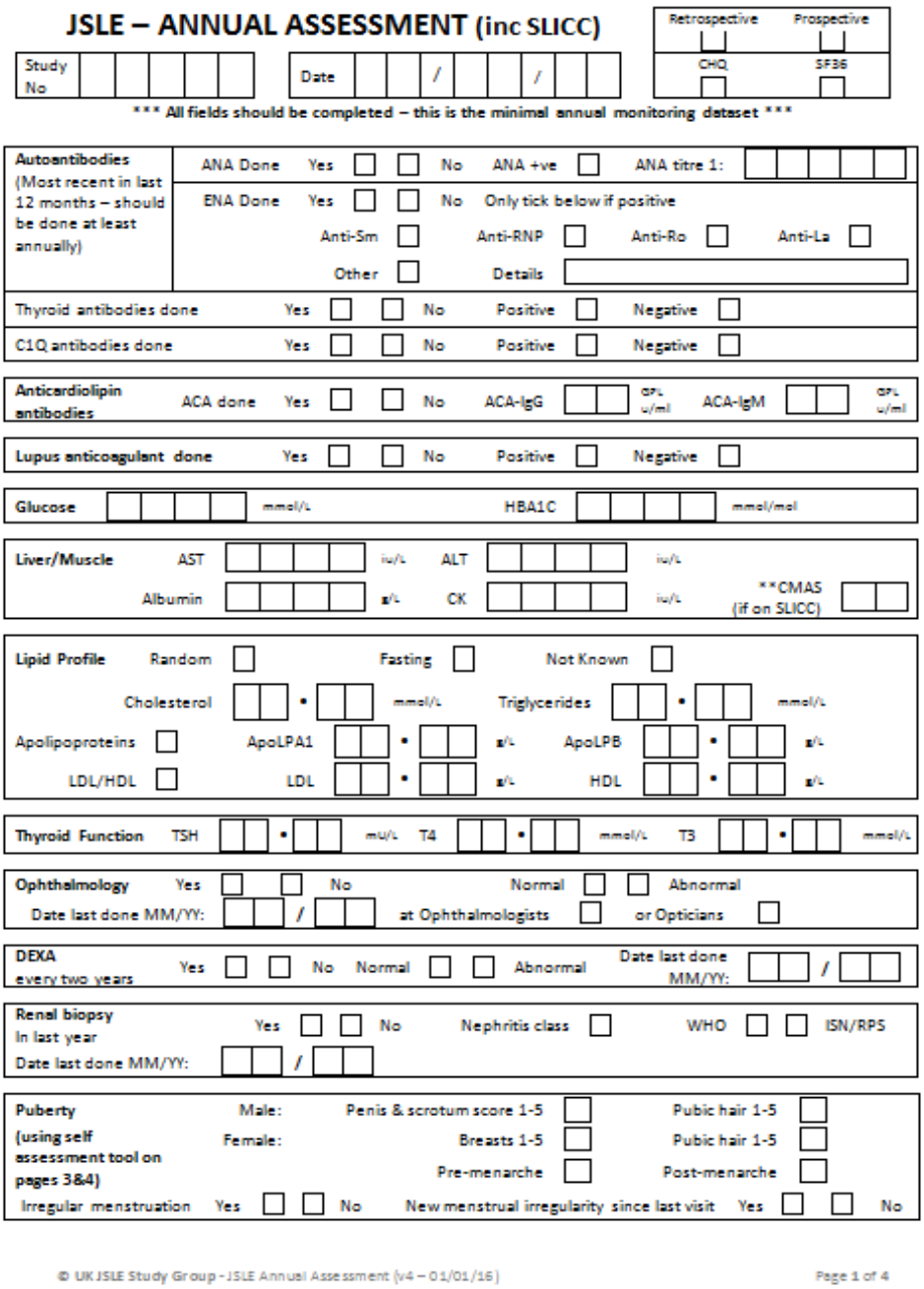
Please note: My working hours are Monday to Friday, **8:00am - 4:00pm**.

Please ensure you are familiar with the Research Integrity Concordat

Appendix B: Clinical and consent forms

APPENDIX B: JSLE CLINICAL AND BILAG FORMS

B1: JSLE annual assessment form



JSLE – ANNUAL ASSESSMENT (inc SLICC)

Retrospective ☐ Prospective ☐ CHQ ☐ SF36 ☐

Study No ☐☐☐☐☐☐ Date ☐☐/☐☐/☐☐

*** All fields should be completed – this is the minimal annual monitoring dataset ***

Autoantibodies (Most recent in last 12 months – should be done at least annually)
ANA Done Yes ☐ No ☐ ANA +ve ☐ ANA titre 1: ☐☐☐☐☐
ENA Done Yes ☐ No ☐ Only tick below if positive
Anti-Sm ☐ Anti-RNP ☐ Anti-Ro ☐ Anti-La ☐
Other ☐ Details ☐

Thyroid antibodies done Yes ☐ No ☐ Positive ☐ Negative ☐

C1Q antibodies done Yes ☐ No ☐ Positive ☐ Negative ☐

Anticardiolipin antibodies ACA done Yes ☐ No ☐ ACA-IgG ☐☐ GPL u/ml ACA-IgM ☐☐ GPL u/ml

Lupus anticoagulant done Yes ☐ No ☐ Positive ☐ Negative ☐

Glucose ☐☐☐☐ mmol/L HBA1C ☐☐☐☐ mmol/mol

Liver/Muscle AST ☐☐☐☐ iu/L ALT ☐☐☐☐ iu/L
Albumin ☐☐☐☐ g/L CK ☐☐☐☐ iu/L **CMAS (if on SLICC) ☐☐

Lipid Profile Random ☐ Fasting ☐ Not Known ☐
Cholesterol ☐☐.☐☐ mmol/L Triglycerides ☐☐.☐☐ mmol/L
Apolipoproteins ☐ ApoLPA1 ☐☐.☐☐ g/L ApoLPB ☐☐.☐☐ g/L
LDL/HDL ☐ LDL ☐☐.☐☐ g/L HDL ☐☐.☐☐ g/L

Thyroid Function TSH ☐☐.☐☐ mU/L T4 ☐☐.☐☐ mmol/L T3 ☐☐.☐☐ mmol/L

Ophthalmology Yes ☐ ☐ No Normal ☐ ☐ Abnormal
Date last done MM/YY: ☐☐/☐☐ at Ophthalmologists ☐ or Opticians ☐

DEXA every two years Yes ☐ ☐ No Normal ☐ ☐ Abnormal Date last done MM/YY: ☐☐/☐☐

Renal biopsy In last year Yes ☐ ☐ No Nephritis class ☐ WHO ☐ ☐ ISN/RPS
Date last done MM/YY: ☐☐/☐☐

Puberty (using self assessment tool on pages 3&4)
Male: Penis & scrotum score 1-5 ☐ Pubic hair 1-5 ☐
Female: Breasts 1-5 ☐ Pubic hair 1-5 ☐
Pre-menarche ☐ Post-menarche ☐
Irregular menstruation Yes ☐ ☐ No New menstrual irregularity since last visit Yes ☐ ☐ No

© UK JSLE Study Group - JSLE Annual Assessment (v4 – 01/01/16) Page 1 of 4

JSLE – ANNUAL ASSESMENT (Page 2 of 2)

To be used with Glossary A blank box will be assumed to = 0. Please tick to confirm	☐ Yes ☐ No

Parameters	SLICC Damage Criteria	0	1	2	3
Ocular (either eye by clinic assessment)	Ocular cataract EVER				
	Retinal change OR optic atrophy				
Neuropsychiatric	Cognitive impairment OR major psychosis				
	Seizures requiring therapy for 6 months				
	Cerebral vascular accident ever (Score 2 if >1), or resection not for malignancy				
	Cranial or peripheral neuropathy (excluding optic)				
	Transverse myelitis				
Renal	Estimated or measured GFR <50%				
	Proteinuria 24h, >=3.5g OR ACR > 1000mg/mm OR > 10mg/mm				
	End stage renal disease (regardless of dialysis or transplantation)				
Pulmonary	Pulmonary hypertension (right ventricular prominence, or loud P2)				
	Pulmonary fibrosis (physical and x-ray)				
	Shrinking lung (x-ray)				
	Pleural fibrosis (x-ray)				
	Pulmonary infarction (x-ray) OR resection not for malignancy				
Cardiovascular	Angina OR coronary artery bypass				
	Myocardial infarction ever (score 2 if >1)				
	Cardiomyopathy (ventricular dysfunction)				
	Valvular disease (diastolic murmur, or a systolic murmur > 3/6)				
	Pericarditis x 6 months or pericardectomy				
Peripheral Vascular	Claudication x 6 months				
	Minor tissue loss (pulp space)				
	Significant tissue loss ever (e.g. loss of digit or limb, resection) (Score 2 if > 1)				
	Venous thrombosis with swelling, ulceration, OR venous stasis				
Gastrointestinal	Infarction or resection of bowel (below duodenum), spleen, liver or gall bladder (Score 2 if > 1)				
	Mesenteric insufficiency				
	Chronic peritonitis				
	Stricture OR upper gastrointestinal tract surgery ever				
	Pancreatic insufficiency requiring enzyme replacement or pseudocyst				
Musculoskeletal	Atrophy or weakness (**if yes please record CMAS on page 1 of this form)				
	Deforming or erosive arthritis (including reducible deformities, excluding avascular necrosis)				
	Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)				
	Avascular necrosis (Score 2 if > 1)				
	Osteomyelitis				
	Ruptured tendons				
Kin	Alopecia				
	Extensive scarring of panniculum other than scalp and pulp space				
	Skin ulceration (not due to thrombosis) > 6 months				
Other	Diabetes (regardless of treatment)				
	Malignancy (excluding dysplasia) (Score 2 if > 1)				
	Premature gonadal failure / secondary amenorrhoea				

PUBERTY SELF ASSESSMENT BOYS

UK JSLE COHORT STUDY NUMBER:

.....

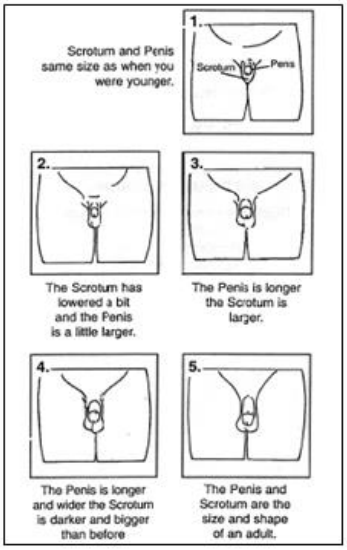
DATE:

.....

PUBERTY SELF ASSESSMENT – BOYS

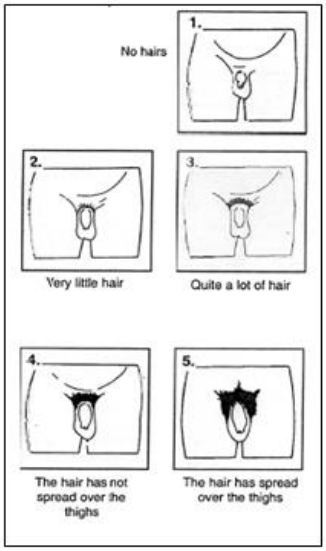
Please look at the **Penis** and **Scrotum** only in these pictures

- Please tick the box that looks most like you now



Please look at the Pubic Hair only in these pictures

- Please put a tick in the box that looks most like you now



PUBERTY SELF ASSESSMENT GIRLS

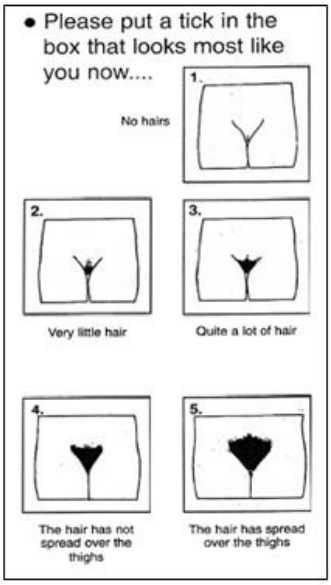
UK JSLE COHORT STUDY NUMBER:

.....

DATE:

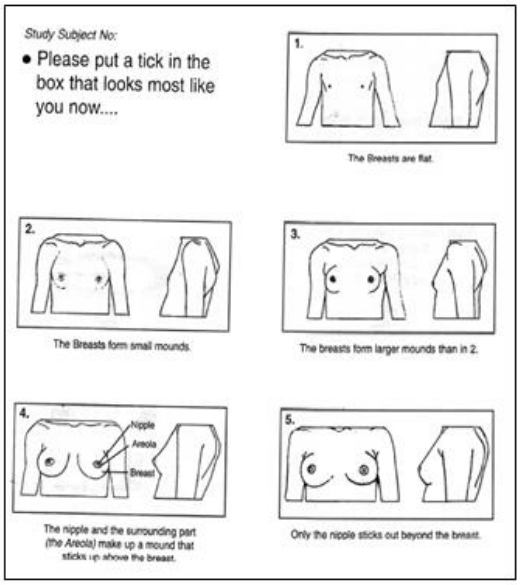
.....

- Please put a tick in the box that looks most like you now....



Study Subject No:

- Please put a tick in the box that looks most like you now....



Please answer the following questions:

1. Have you started having your period yet? Y / N
2. If yes, at what age did it start:
3. Do you have irregular periods? Y / N
4. If yes, is this a new problem since your last clinic appointment? Y/N

B2: JSLE ACR and SLICC form

SLICC CLASSIFICATION CRITERIA AT ANNUAL REVIEW

JSLE – ACR CLASSIFICATION CRITERIA AT ANNUAL REVIEW

Study No						Date			/			/			Retrospective ☐	Prospective ☐

These are cumulative. Please add new criteria only or tick the box if no new criteria since last review ☐

Criterion		Present	Subtype		Definition
1	Malar Rash	☐			Fixed erythema, flat or raised, over malar eminences, tending to spare the nasolabial folds
2	Discoid Lupus	☐			Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	☐			Skin rash as a result of the unusual reaction to sunlight, by patient history or physician observation
4	Oral/nasal ulcerations	☐			Oral or nasopharyngeal ulceration, usually painless, observed by physician
5	Non-erosive Arthritis	☐			Non-erosive arthritis involving 2 or more peripheral joints , characterised by tenderness, swelling or effusion
6	Serositis	☐	A	☐	Pleuritis-convincing history of pleuritic pain or rub heard, or evidence of pleural effusion
		☐	B	☐	Pericarditis–documented ECG or rub, or evidence of pericardial effusion
7	Nephritis	☐	A	☐	Persistent proteinuria > 0.5g/day or > +++ (on protein dipstix) if quantification not performed
			B	☐	Cellular casts – may be red cell, haemoglobin, granular, tubular or mixed
8	Neurologic	☐	A	☐	Seizures in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)
			B	☐	Psychosis in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)
9	Haematological Disorder	☐	A	☐	Haemolytic anaemia with reticulocytes
			B	☐	Leucopenia < 4,000/mm^3 total on 2 or more occasions
			C	☐	Lymphopenia < 1,500/mm^3 on 2 or more occasions
			D	☐	Thrombocytopenia < 100,000/mm^3 in absence of offending drugs
10	Immunological Disorder	☐	A	☐	Anti-DNA: antibody to native DNA in abnormal titre
			B	☐	Anti-Sm: presence of antibody to Sm nuclear antigen
			C	☐	+ve finding of anti-phospholipid antibodies based on:
				C1 ☐	Abnormal level of IgG or IgM anti-cardiolipiantibody
				C2 ☐	Positive test result for lupus anticoagulant (standard method)
				C3 ☐	False +ve serologic result for syphilis > 6 mths
11	ANA	☐			Abnormal titre ANA at any time point in absence of drugs known to be associated with "drug-induced lupus"

If less than 4 criteria why evolving Lupus?	

Study No								Date			/			/		
These are cumulative. Please add new criteria only or tick the box if no new criteria since last review																☐

		Criterion	Present	Subtype		Definition
Clinical Criteria	1	Acute cutaneous lupus *in the absence of dermatomyositis*	☐	A	☐	Lupus malar rash (do not count if malar discoid)
				B	☐	Bullous lupus
				C	☐	Toxic epidermal necrolysis variant of SLE
				D	☐	Maculopapular lupus rash
				E	☐	Photosensitive lupus rash
					☐	Subacute cutaneous lupus (Non-indurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with post-inflammatory dyspigmentation or telangiectasias)
	2	Chronic cutaneous lupus	☐	A	☐	Classic discoid rash localised (above the neck)
				B	☐	Classic discoid rash generalised (above and below the neck)
				C	☐	Hypertrophic (verrucous lupus)
				D	☐	Lupus panniculitis (profundus)
				E	☐	Mucosal lupus
				F	☐	Lupus erythematosus tumidus
				G	☐	Chilblains lupus
				H	☐	Discoid lupus / lichen planus overlap
	3	Ulcers	☐	A	☐	Oral ulcers (Palate/Buccal/Tongue) *in absence of other causes, such as vasculitis, Behcet's disease, infection (herpes virus), inflammatory bowel disease, reactive arthritis and acidic foods*
				B	☐	Nasal ulcers - *in the absence of other causes, such as vasculitis, Behcet's disease, infection (herpes virus), inflammatory bowel disease, reactive arthritis and acidic foods*
	4	Non-scarring alopecia	☐			Diffuse thinning or hair fragility with visible broken hairs *in the absence of other causes such as alopecia areata, drugs, iron deficiency and androgenic alopecia*
	5	Synovitis	☐			Involving 2 or more joints, characterised by swelling or effusion OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness
	6	Serositis	☐	A	☐	Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub
				B	☐	Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by ECG *in the absence of other causes such as infection, uraemia, and Dressler's pericarditis*
	7	Renal	☐	A	☐	Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 500mg protein/24 hours
				B	☐	Red blood cell casts
	8	Neurological	☐	A	☐	Seizures
				B	☐	Psychosis
				C	☐	Mononeuritis multiplex *in the absence other known causes such as primary vasculitis*
				D	☐	Myelitis
				E	☐	Peripheral or cranial neuropathy *in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus*
				F	☐	Acute confusional state *in the absence of other causes, including toxic/metabolic, uraemia, drugs*
	9	Haemolytic anaemia	☐			
	10	Leukopenia or lymphopenia	☐	A	☐	Leukopenia (<4,000/mm³ at least once) *in the absence of other known causes such as Felty's syndrome, drugs and portal hypertension*
				B	☐	Lymphopenia (<1,000/mm³ at least once) *in the absence of other known causes such as corticosteroids, drugs and infection*
	11	Thrombocytopenia	☐			Thrombocytopenia <100,000/mm³ at least once *in the absence of other known causes such as drugs, portal hypertension , and thrombotic thrombocytopenic purpura*
Immunologic criteria	1	ANA	☐			ANA level above laboratory reference range
	2	Anti-dsDNA	☐			Anti-dsDNA antibody level above laboratory reference range (or >2-fold the reference range if tested by ELISA)
	3	Anti-Sm	☐			Presence of antibody to Sm nuclear antigen
	4	Antiphospholipid antibody positivity	☐	A	☐	Positive test result for lupus anticoagulant
				B	☐	False-positive test result for rapid plasma reagin
				C	☐	Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)
				D	☐	Positive test result for anti-β2-glycoprotein I (IgA, IgG, or IgM)
	5	Low complement	☐	A	☐	Low C3
				B	☐	Low C4
				C	☐	Low CH50
	6	Direct Coombs' test	☐			*in the absence of haemolytic anaemia*

JSLE pBILAG form

JSLE – BILAG Form

Routine	Flare
☐	☐
Retrospective	**Prospective**
☐	☐

Study No ☐☐☐☐☐☐ Date ☐☐/☐☐/☐☐

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new;; 0 = not present (except for Y/N and numeric questions).

Height cm ☐☐☐☐ **Weight** kg ☐☐☐☐ **Systolic BP** mmHg ☐☐☐ **Diastolic BP** mmHg ☐☐☐

Urinalysis (score 0, trace, 1+, 2+, 3+). Not Done ☐
If abnormal urinalysis send urine for Microscopy, culture, sensitivities (MCS)

Proteinuria ☐ Haematuria ☐ Leucocytes ☐ Nitrites ☐ Menstruating Y / N

NB. The following bloods should be sent as part of BILAG: FBC, ESR, CRP, C3, C4, dsDNA, total Igs, Creatinine, urinary protein/Cr or Alb/Cr ratio. **Please tick to confirm done** ☐ Yes ☐ No

To be used with Glossary: * = Definition in Glossary
Only features attributable to JSLE to be recorded and refer only to last 4 week compared with previous 4 weeks

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions). **A blank box will be assumed to = 0 or No. Please tick to confirm** ☐ Yes ☐ No

General		
* 1	Pyrexia (documented >37.5°C)	
* 2	Weight Loss – unintentional; >5%	
* 3	Lymphadenopathy/splenomegaly	
4	Fatigue/malaise/lethargy	
5	Anorexia	
Mucocutaneous		
* 6	Skin eruption – severe active (not discoid/bullous/panniculitis)	
* 7	Skin eruption – mild	
* 8	Active discoid lesions: generalised/extensive	
* 9	Active discoid lesions: localised include lupus profundus	
* 10	Alopecia (severe, active)	
* 11	Alopecia (mild)	
* 12	Panniculitis/bullous lupus (severe)	
*13	Panniculitis/bullous lupus (mild)	
* 14a	Angio-oedema (severe)	
* 14b	Angio-oedema (mild)	
* 15	Mucosal ulceration (severe)	
*16	Mucosal ulcers (mild)	
* 17	Malar erythema	
18	Subcutaneous nodules	

19	Perniotic skin lesions				
*20	Peri-ungual erythema / chillblains				
21	Swollen fingers	Y			N
22	Sclerodactyly	Y			N
23	Calcinosis	Y			N
24	Telangiectasia	Y			N
25	Splinter haemorrhages	Y			N
Neurological					
26	Impaired level of consciousness				
* 27	Cognitive dysfunction				
* 28	Acute psychosis or delirium or confusional state				
* 29	Psychosis				
* 30	Seizure disorder				
* 31	Status epilepticus				
* 32	Cerebral vascular disease (not due to vasculitis)				
* 33	Cerebral vasculitis				
* 34	Aseptic meningitis				
* 35	Mononeuropathy (single/multiplex)				
36	Ascending or transverse myelitis				
* 37	Demyelinating syndrome				
* 38	Myelopathy				

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new;; 0 = not present (except for Y/N and numeric questions).

Neurological continued						
* 39	Acute inflammatory demyelinating polyradiculoneuropathy					
40	Peripheral neuropathy					
* 41	Cranial neuropathy					
* 42	Plexopathy					
* 43	Polyneuropathy					
* 44	Autonomic disorder					
* 45	Disc swelling					
* 46	Chorea					
* 47	Cerebellar ataxia (isolated)					
* 48	Movement disorder					
* 49	Lupus headache (severe, unremitting)					
* 50	Episodic migrainous headaches					
* 51	Tension headache					
* 52	Cluster headache					
* 53	Headache from IC hypertension					
* 54	Organic depressive illness					
* 55	Mood disorder (depression/mania)					
* 56	Anxiety disorder					
* 57	Organic brain syndrome					
Musculoskeletal						
* 58	Definite myositis (severe)					
* 59	Myositis with incomplete criteria					
* 60	Myositis (mild)					
* 61	Myalgia					
* 62	Severe polyarthritis – with loss of function					
* 63	Moderate arthritis					
* 64	Arthralgia					
* 65	Tendonitis/tenosynovitis					
66	Tendon contractures and fixed deformity	Y				N
67	Aseptic necrosis	Y				N
Cardiovascular & Respiratory						
* 68	Pleuropericardial pain					
* 69	Dyspnoea					
* 70	Cardiac failure					
* 71	Friction rub					
* 72	Effusion (pericardial or pleural)					
* 73	Mild or intermittent chest pain					
74	Progressive CXR changes – lung fields	Y				N
75	Progressive CXR changes – heart size	Y				N
76	ECG evidence of pericarditis/ myocarditis/endocarditis	Y				N
* 77	Cardiac arrhythmia including tachycardia (>100 no fever)	Y				N
* 78	Pulmonary function fall by >20%	Y				N
79	Cytohistological evidence of inflammatory lung disease	Y				N
* 80	Myocarditis - mild					
* 81	New valvular dysfunction					
* 82	Cardiac tamponade					
* 83	Pleural effusion with dyspnoea					
* 84	Pulmonary haemorrhage/vasculitis					
* 85	Interstitial alveolitis/pneumonitis					
* 86	Shrinking lung syndrome					
* 87	Aortitis					
* 88	Coronary vasculitis					
Vasculitis						
* 89	Major cutaneous vasculitis including ulcers					
* 90	Major abdominal crisis due to vasculitis					
91	Recurrent thromboembolism (excluding strokes)					
92	Raynaud's					
93	Livido reticularis					

JSLE – BILAG Form

Study No							Date			/			/		

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Vasculitis continued					
94	Superficial phlebitis				
* 95	Minor cutaneous vasculitis (nailfold, digital, purpura, urticaria)				
96	Thromboembolism (excl stroke) 1st episode	Y			N
Renal					
* 99	Severe hypertension	Y			N
103	Newly documented proteinuria (level 4)	Y			N
* 104	Nephrotic syndrome	Y			N
* 108	Histological evidence of active nephritis within 3 months	Y			N
Gastrointestinal					
* 109	Lupus peritonitis				
* 110	Abdominal serositis or ascites				
* 111	Lupus enteritis/colitis				
* 112	Malabsorption				
* 113	Protein losing enteropathy				
* 114	Intestinal pseudo-obstruction				
* 115	Lupus hepatitis				

* 116	Acute lupus cholecystitis	
* 117	Acute lupus pancreatitis	
Ophthalmic		
* 118	Orbital inflammation with myositis and/or proptosis	
* 119	Keratitis – severe	
* 120	Keratitis – mild	
121	Anterior uveitis	
* 122	Posterior uveitis/retinal vasculitis – severe	
* 123	Posterior uveitis/retinal vasculitis – mild	
124	Episcleritis	
* 125	Scleritis – severe	
* 126	Scleritis – mild	
* 127	Retinal / choroidal vaso-occlusive disease	
* 128	Isolated cotton-wool spots (cytoid bodies)	
* 129	Optic neuritis	
* 130	Anterior ischaemic optic neuropathy	

Function

Patient CHAQ ☐ • ☐☐

Global assessment on CHAQ (0: Very well – 100: Very ill ☐☐☐

Physicians clinical global score — Very well 0 ——— 100 Very ill

Important assessment of current clinical status

In relation to the patients LUPUS ACTIVITY at this time point, please tick the box that best describes your opinion as to their current flare status:

1. Clinical improvement – no clinical flare ☐
2. No change in Lupus activity ☐
3. Minor flare (may not require specific therapy) ☐
4. Moderate flare ☐
5. Major flare ☐

A flare of JSLE has been defined as "a measurable worsening of SLE disease activity in at least one organ system, involving new or worse signs of disease that may be accompanied by new or worse JSLE symptoms; depending on the severity of the flare, more intensive therapy may be required" [1]

1. Brunner HI, Klein-Gitelman MS, Higgins GC, et al. Arthritis Care Res. 2010;62(6):811-20.

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new,; 0 = not present (except for Y/N and numeric questions).

Treatment	Current dose	Revised dose
Hydroxychloroquine (mg/day)	☐☐☐	☐☐☐
Azathioprine (mg/day)	☐☐☐	☐☐☐
Mycophenolate (mg/day)	☐☐☐☐	☐☐☐☐
Cyclosporin (mg/day)	☐☐☐	☐☐☐
Prednisolone (mg/day)	☐☐.☐	☐☐.☐
Methotrexate (mg/week)	☐☐.☐	☐☐.☐
	Oral ☐	Oral ☐
	Subcut ☐	Subcut ☐

	Current dose	Total number of pulses since last visit
IVIG (g/pulse)	☐☐☐.☐	☐☐

Rituximab:		**Cyclophosphamide:** IV ☐ Oral ☐	
Total dose per cycle (mg)	☐☐☐☐☐	Total dose since last visit (mg)	☐☐☐☐☐
Number of infusions per cycle	☐☐	Number of infusions since last visit	☐☐
Number of cycles since last visit	☐☐	Cumulative dose	☐☐☐☐☐
IV methyl-prednisolone in last 3 months	<5 pulses ☐	>=5 pulses ☐	

Other Drugs

Aspirin ☐	Bisphosphonate ☐	Oral contraceptive pill ☐					
Angiotensin receptor blocker ☐	Ca** blockers ☐	Statins ☐					
Other biological DMARDS ☐	ACEi ☐	Diuretic ☐	Anticoagulant ☐				

Clinicians Intention re Medication

Please tick which of the following best describes your intention to change treatment, and explain why:

1) Decrease in treatment ☐
- Disease improvement ☐
- Side effects of treatment ☐
- Compliance problems ☐
- Weaning regimen ☐

2) Increase in treatment ☐
- Disease worsening ☐
- Standard dose increment ☐
- Dose increment due to weight ☐

3) Change in DMARD ☐
- Concerns over efficacy ☐
- Planned maintenance treatment ☐
- Side effects of treatment ☐
- Compliance problems ☐

4) No change in treatment ☐
- Active disease (induction phase) ☐
- Stable, not yet decrease ☐
- Patients choice ☐
- Not on Medication ☐

JSLE – BILAG Form

Study No | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Results Page

Microscopy & culture results

MCS results	1. Proven UTI (>1x10^5 growth (cfu) of a single organism)	Y / N	2. Mixed growth / contamination	Y / N
	3. Microscopy results: a) White cell count	(per hpf)	b) Red cell count	(per hpf)
	c) Red cell casts	Y / N	d) White cell casts	Y / N

Renal function

* 102a	Urinary Alb/Cr ratio (mg/mmol Cr)		105	Creatinine (plasma/serum)	
* 102b	Urinary Protein/Cr Ratio (mg/mmol Cr)		106a	GFR:EDTA clearance (exact) (mls/min. 1.73m^2)	
102c	24hr/Urinary Protein(g)		106b	GFR: ht/creat ratio (estimate)	
	Not done		107	Active urinary sediment	Y N

Haematology

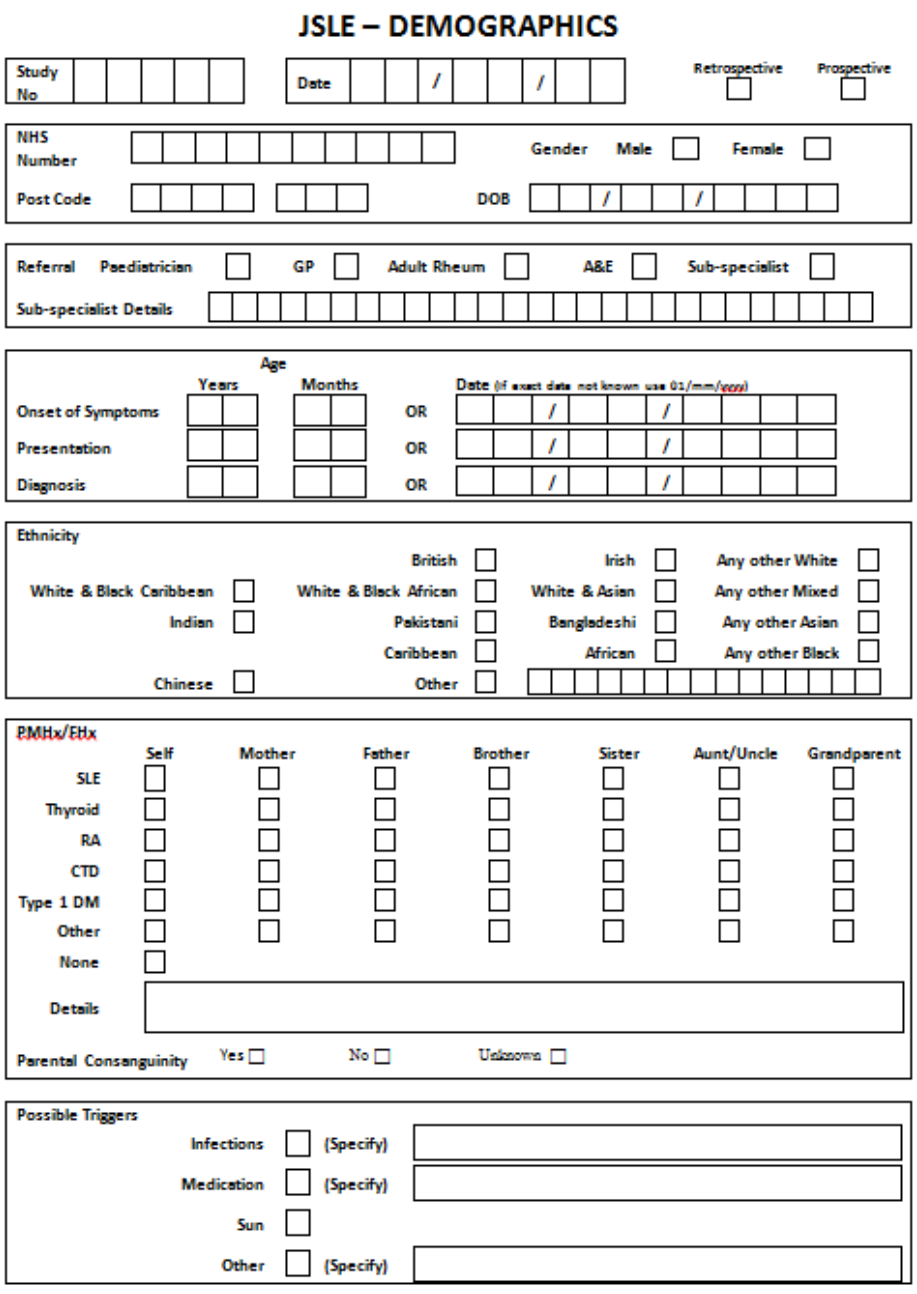
* 131	Haemoglobin g/dl		* 135	Platelets x 10^9/l	
* 132	Total white cell count x 10^9/l		* 136	Evidence of active haemolysis	Y N
* 133	Neutrophils x 10^9/l		137	Coomb's test positive	Y N
134	Lymphocytes x 10^9/l		* 138	TTP	Y N

Other measures of disease activity

ESR (mm/hr)		dsDNA	
CRP		IgG	
C3 (g/l)		IgA	
C4 (g/l)		IgM	
Viscosity (mPa.s) If no ESR		Ferritin (µg/L)	

APPENDIX C: DEMOGRAPHIC FORMS

C1: JSLE patient demographic form



JSLE – DEMOGRAPHICS

Study No | Date / / | Retrospective ☐ | Prospective ☐

NHS Number | Gender Male ☐ Female ☐

Post Code | DOB / /

Referral Paediatrician ☐ GP ☐ Adult Rheum ☐ A&E ☐ Sub-specialist ☐

Sub-specialist Details

	Age Years	Months		Date (if exact date not known use 01/mm/yyyy)
Onset of Symptoms			OR	/ /
Presentation			OR	/ /
Diagnosis			OR	/ /

Ethnicity

	British ☐	Irish ☐	Any other White ☐
White & Black Caribbean ☐	White & Black African ☐	White & Asian ☐	Any other Mixed ☐
Indian ☐	Pakistani ☐	Bangladeshi ☐	Any other Asian ☐
	Caribbean ☐	African ☐	Any other Black ☐
Chinese ☐	Other ☐		

PMHx/FHx

	Self	Mother	Father	Brother	Sister	Aunt/Uncle	Grandparent
SLE	☐	☐	☐	☐	☐	☐	☐
Thyroid	☐	☐	☐	☐	☐	☐	☐
RA	☐	☐	☐	☐	☐	☐	☐
CTD	☐	☐	☐	☐	☐	☐	☐
Type 1 DM	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐
None	☐						

Details

Parental Consanguinity Yes ☐ No ☐ Unknown ☐

Possible Triggers

Infections ☐ (Specify)

Medication ☐ (Specify)

Sun ☐

Other ☐ (Specify)

© UK JSLE Study Group - JSLE Demographics (v4 – 01/01/16)

C2: Paediatric control patient demographic form

CONTROL DEMOGRAPHICS

Study Number: _____ Gender: Male ☐ Female ☐

DOB: __ / __ / ____ Post Code: _____ Date Consented: __/ __ / ____

Ethnicity (please tick)

White British		White & Black Caribbean		Indian		Caribbean		Chinese	
White Irish		White & Black African		Pakistani		African		Other: _____	
Any other white background		White & Asian		Bangladeshi		Any other Black background			
		Any other mixed background		Any other Asian background					

Type of Control

JIA		Renal		Non Inflammatory	

Diagnosis (see list on next page)

_____

(JIA only)

	Age (Years/Months)		Date
Onset of Symptoms	_____	OR	__/__/____
Presentation	_____	OR	__/__/____
Diagnosis	_____	OR	__/__/____

Has there been a family history of SLE, Thyroid problems, RA, CTD, IDDM, renal disease or any other related diseases? Yes ☐ No ☐

	Renal Disease	Autoimmune Disease	Other
Mother			
Father			
Brother			
Sister			
Aunt / Uncle			
Grandparents			

Diagnosis

JIA:	Systemic Oligoarthritis Oligoarthritis (Extended) Oligoarthritis (Persistent) Polyarthritis Polyarthritis (Rheumatoid Factor Positive) Polyarthritis (Rheumatoid Factor Negative) Psoriatic Enthesitis related Other – meeting more than one criteria Other – not meeting other JIA criteria Undetermined
Renal:	Diabetic nethropathy Nephrotic syndrome HSP nephritis Renal dysplasia Renal scarring Other
Non Inflammatory	Minor operation Dental surgery Infusion Other

APPENDIX D: CONSENT FORMS AND INFORMATION SHEETS

D1: JSLE patient/parent information sheet

UK Children's Lupus Study

Information sheet for Parents

(JSLE patients- Liverpool only)

Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare children with Lupus to children without. Specifically we want to compare blood cells to see why Lupus cells are reacting against themselves. We are also looking at urine to help understand the Kidney problems seen.

This project is part of a UK wide study of children with Lupus.

Why are we asking your child to take part?

Because they have Lupus.

Does your child have to take part?

No, it's up to you and your child. Either of you can change your mind at any point without giving a reason. This will not affect their care.

What will your child be asked to do?

- Your child's doctor will collect information about your child's age, gender and how they are when visiting hospital.
- When your child is having their usual blood tests we will collect a little extra blood (2-3 teaspoons) to:
  - Measure antibodies and white cells (these fight infection)
  - Study the genetics of Lupus
  - Study the Kidney involvement
- If your child has only just been diagnosed we will collect an extra sample of blood before starting treatment and at one week, one month and three months of starting treatment.
- We will also ask if your child can provide a urine sample.
- When Lupus affects the kidney, it may be necessary for your child to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. For this study, we would ask to be able to use any extra sample which is left over after all the standard tests have been done. This sample would be labelled with your child's study code and stored at the University of Liverpool, Alder Hey Children's Hospital. DNA (the chemical which contains your genes) will also be extracted from this sample.
- If your child has previously had a kidney biopsy we will ask your local hospital if they have enough to provide us with a piece of this.
- We will tell your child's GP they are helping us with the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for an email address
- We will be in touch about future studies in Lupus to see if your child is interested in taking part.

Will it do some good if you say yes?

By doing experiments on the samples provided, your child will help us to improve understanding of Lupus and to create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and your child will not feel any different. Helping us with the study will not affect your child's hospital care.

What will happen to the results of the research study?

Everything we discover will be published in medical journals. Your child will not be identified in any way. Your child's test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

The research is funded by the Alder Hey Children's NHS Foundation Trust and its Research and Development Department, the charity Lupus UK and The Alder Hey Kidney Fund.

No one receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What will happen to the information collected?

- All information and samples collected will be strictly confidential and anonymised.
- All your child's forms will be kept in the hospitals research office in a locked filing cabinet. We will keep your child's name and hospital details on a list in the research office so we know they are in the study. The office will be locked when non-attended.
- All information kept on study computers (in offices of the UK JSLE Study Group at the University of Liverpool) will only record data using a study number and will be strictly confidential. Details identifying your child will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your child's doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

D2: JSLE patient/parent consent form

Alder Hey Children's NHS
NHS Foundation Trust

Parental Consent Form

(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 – 1st January 2016) for the above study and have had the chance to ask questions	
2.	I understand my child's taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected	
3.	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my child's blood may be used to investigate the immune system	
5.	I agree that a small amount of my child's blood may be collected and then gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my child's genes will be fed back to them or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if my child were to require a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital	
8.	**To be completed if your child has had a kidney biopsy in the past:** I agree that a small amount of left over kidney tissue from my child's previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy sample my child provides. I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree to allow researchers to make contact with me and my child about other studies or a follow-up of this study through my child's doctors and my child's NHS number	
11.	I give permission for my child's GP to be informed that information about my child is to be held on the study database	

12.	I agree to my child being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
13.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
14.	I agree to provide an email address. I understand that this will be stored securely. I understand it will only be used to provide information about the study such as the newsletter and to provide a way for the study group to contact us for follow up in the future.	
15.	I agree for my child to take part in the above study	

Name of patient		
Name of person with parental responsibility for patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

D3: JSLE patient information sheets

JSLE patient information sheet for under 6

UK Children's Lupus Study

Information sheet which can be read to patients aged less than 6 years

(JSLE patients – All centres, including Liverpool & GOSH)

Note to reader: Please read the whole sheet to yourself before reading it to your child

Your doctor has told you that you have Lupus.

Doctor's want to find out why some children get Lupus, and others don't.

By finding out more about why children get Lupus we hope to be able to make better medicines.

We are doing this all over the country with children who have Lupus.

What will happen if I say yes?

- We would collect information about how you are
- We will take a small sample of your blood (2-3 teaspoons) when it is already being taken. There are NO extra needles!
- We will also ask for a urine sample

Do I have to help?

No, not if your mum or dad or the grown up looking after you decide that they don't want you to. You will be looked after just the same.

Will it do some good if I say yes?

Yes you will be helping us make better treatments for Lupus

What do I have to do now?

We are asking you to say "Yes" or "No" to the question:

"Can we collect information about how you are and a small blood and urine sample?"

What if I have questions?

If you have any questions you or your mum and dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!

JSLE patient information sheet for 6-12

UK Children's Lupus Study

Information sheet for patients aged 6-12 years

(JSLE patients – Liverpool & GOSH only)

Why is this being done?

This research is looking at what causes Lupus. We don't fully understand this, especially in children.

We hope that by understanding more about the causes of Lupus we can make better treatments.

To do this we need to compare children with Lupus, like you, to children without Lupus.

Why have I been chosen?

You are being asked to take part in this study because you have Lupus.

What will happen if I say yes?

We will ask you to write your name on a form. This is to say that you understand what the study is about and what will happen.

We would like to collect information about how you are.

We will take a small sample of your blood (2-3 teaspoons) when it is already being taken. There are NO extra needles!

We will also ask for a urine sample.

If Lupus affects your kidneys your doctor may have done / or be planning to do some tests on a little bit of your kidney tissue. We would ask your doctor for a bit of 'left over' kidney tissue as part of this study.

Do I have to help?

No, not if your mum or dad or the grown up looking after you decide that they do not want you to. You will be looked after just the same.

Will it do some good if I say yes?

By looking at the samples given, you will be helping us to treat children with Lupus, like you, better in the future.

Will anything bad happen to me if I take part?

No, nothing bad will happen to you. All information that is collected about you will be kept private between you / your parents and the study organisers.

What do I have to do now?

If you want to help, you and your mum, or dad or the grown up looking after you will have to say it's ok.

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!

JSLE patient information sheet for 13-15

UK Children's Lupus Study

Information sheet for patients aged 13-15 years |

(JSLE patients - Liverpool only)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells and urine of children with and without Lupus. This will give us information on why Lupus cells are reacting against themselves and the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are.
- When you are having your usual blood tests we will collect a little extra blood (2-3 teaspoons). We will use this to:
  - Measure your autoantibodies and white cells (these usually fight infection)
  - Study the genetics of Lupus
  - Study the kidney involvement
- If you have only just been diagnosed we will collect an extra sample of blood before you start treatment and after one week, one month and three months of starting treatment.
- We will also ask if you can give us a urine sample.
- When Lupus affects the kidney, it may be necessary to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. We would like to have any extra sample, which is left over after all the standard tests have been done. This sample would be stored at the University of Liverpool. DNA (contained in your genes) in the kidney sample will also be tested.
- If you have had a kidney biopsy in the past we will ask your local hospital if they have enough to provide us with a small piece of this.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect health information on all people in the UK e.g. if someone dies or develops cancer.
- We will ask for an email address
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years.

Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group".

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department, Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospitals research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What if I have questions?

If you have any questions you, your mum / dad or the grown up looking after you can speak to the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

JSLE patient information sheet for 16 and over

UK Children's Lupus Study

Information sheet for patients aged 16 years and older

(JSLE patients - Liverpool only)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with and without Lupus, to see why Lupus cells are reacting against themselves. We are also looking at urine to better understand the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are when you visit the hospital.
- When you are having your usual blood tests we will collect a little extra blood (2-3 teaspoons). We will use this to:
  - Measure your autoantibodies and white cells (these usually fight infection)
  - Study the genetics of Lupus
  - Study the kidney involvement
- If you have only just been diagnosed we will collect an extra sample of blood before you start treatment and after one week, one month and three months of starting treatment.
- We will also ask if you can give us a urine sample.
- When Lupus affects the kidney, it may be necessary to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. We would like to have any extra sample which is left over after all the standard tests have been done. This sample would be stored at the University of Liverpool, Alder Hey Children's Hospital. DNA (contained in your genes) in the kidney sample will also be tested.
- If you have had a kidney biopsy in the past we will ask your local hospital if they have enough to provide us with a small piece of this.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for an email address
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus and create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years. Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's Hospital.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department, Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospitals research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

D4: JSLE patient assent form

Alder Hey Children's NHS
NHS Foundation Trust

Assent Form – Patients

(Liverpool and GOSH only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have been told about the study and given the information sheet (Version 4 – 1st January 2016) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and I can stop taking part at any time, without giving any reason, without this making any difference to my medical care or legal rights	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust, where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood and in some cases urine may be used for research.	
5.	I agree that if I need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
6.	**To be completed by patients who have had a kidney biopsy in the past:** I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
7.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
8.	I agree that I will take part in the above study	
9.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and NHS number	
10.	I give permission for my GP to be informed that information about me is to be held on the study database	
11.	I agree to being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
12.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	

13.	I agree to provide my email address. I understand that this will be stored securely. I understand it will only be used to provide me with information about the study such as the newsletter and to provide a way for the study group to contact me for follow up in the future.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

D5: JSLE patient consent form

Alder Hey Children's NHS
NHS Foundation Trust

Patient's Consent Form

(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 -1st January 2016) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood may be used to investigate the immune system	
5.	I agree that a small amount of my blood may be collected and gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my genes will be fed back to me or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if I was to need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
8.	**To be completed by patients who have had a kidney biopsy in the past;** I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and my NHS number	
11.	I give permission for my GP to be informed that information about me is to be held on the study database	

12.	I agree to being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
13.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
14.	I agree to provide my email address. I understand that this will be stored securely. I understand it will only be used to provide me with information about the study such as the newsletter and to provide a way for the study group to contact me for follow up in the future.	
15.	I agree that I will take part in the above study	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

D6: Paediatric control patient/parent information sheet

UK Children's Lupus Study

Information sheet for Parents

(Paediatric controls - Liverpool only)

Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare children with Lupus to children without Lupus, like your child. Specifically we need to compare blood cells to see why Lupus cells are reacting against themselves and urine to detect inflammation of the kidney, a common complication of Lupus.

This project is part of a UK wide study of children with Lupus.

Why are we asking your child to take part?

Because they do not have Lupus.

Does your child have to take part?

No, it's up to you and your child. You and your child can change your mind at any point without giving a reason. This will not affect the care they receive.

What will your child be asked to do?

- We will take a sample of your child's blood (2-3 teaspoons). If your child needs a blood test for another reason we will take it at the same time.
- We will also ask if your child can provide a urine sample. We use this to look at how the kidneys work in Lupus.
- We will collect information about your child's age, gender and your child's general health. Your child's participation in the study will be kept confidential.
- We will keep your child's name and hospital details on a list in the hospitals locked research office so we know you are in the study.
- We will tell your GP you are helping us with the study

Will it do some good if we say yes?

By doing experiments on the samples given by your child, this will help us create better treatments for children with Lupus.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect their care.

What will happen to the results of the research study?

Everything we discover will be presented at scientific meetings and published in medical journals. Your child will not be identified in any way.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department at Alder Hey, the charity Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What will happen to the information collected?

All information and samples collected will be strictly confidential and anonymised.

Forms will be kept in the hospitals research office. All forms will be stored in locked filing cabinets in rooms that are locked when non-attended.

All information kept on study computers (in offices of the UK JSLE Study Group at the Institute Child Health, University of Liverpool) will only record data using a study number and will be strictly confidential. Details identifying your child will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

D7: Paediatric control patient/parental consent form

Alder Hey Children's NHS
NHS Foundation Trust

Parental Consent Form
(Paediatric Controls - Liverpool only)
UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 – 1st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand my child's taking part in voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected.	
3.	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my child's blood may be used to investigate Lupus.	
5.	I agree that a small amount of my child's urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree for my child to take part in the above study	

Name of patient		
Name of person with parental responsibility for patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

D8: Paediatric control patient information sheet

Paediatric control patient information sheet for under 6

UK Children's Lupus Study

Information sheet for patients aged less than 6 years

(Paediatric controls - Liverpool only)

Note to reader: Please read the whole sheet to yourself before reading it to your child

Some children are sick because they have an illness called Lupus.

Doctor's want to find out why some children get Lupus and others don't

To do this we need to compare children with Lupus to children without Lupus, like you

Why have I been chosen?

Because you do not have Lupus

What will happen if I say yes?

- We would like to collect information about how you are.
- We will take a small sample of your blood (2-3 teaspoons) when it is already being taken or you are going for an operation. There are NO extra needles!
- We will also ask for a urine sample.

Do I have to help?

No, not if you or your mum / dad or the grown up looking after you decide you do not want to. You will be looked after just the same.

Will it do some good if I say yes?

Yes, you will be helping us invent better treatments for sick children with Lupus

What do I have to do now?

We are asking you to say "Yes" or "No" to the question:

"Can we collect information about how you are and a small blood and urine sample?"

What if I have questions?

If you have any questions you or you mum or dad, or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!

Paediatric control patients' information sheet for 6-12

UK Children's Lupus Study

Information sheet for patients aged 6-12 years

(Paediatric controls - Liverpool only)

Why is this research being done?

This research looks at a condition called "Lupus". We want to understand more about what causes it and the best ways to treat it.

To do this we need to compare children with Lupus to children without Lupus.

Why have I been chosen?

You are being asked to take part in this study because you do not have Lupus.

Do I have to help?

No, not if you, your mum / dad or the grown up looking after you do not want to. You will be looked after just the same.

What will happen if I say yes?

You will be asked to write your name on a form. This is to say that you understand the study and what will happen. You will be given your own copy of the form to keep as well as this leaflet.

We will take a sample of your blood (2-3 teaspoons) when you are going for your operation. There are NO extra needles!

We will also ask if you can give us a urine sample. We use this to look at how the kidneys work in Lupus.

Will it do some good if I say yes?

Using your samples, we will work on ways to us create better treatments for children with Lupus.

Will anything bad happen to me if I take part?

No, nothing bad will happen to you.

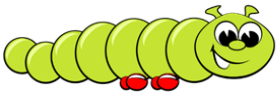
What do I have to do now?

If you want to help, you and your mum or dad or the grown up looking after you will have to say it's ok.

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!



Paediatric control patient information sheet for 13-15

UK Children's Lupus Study

Information sheet for patients aged 13-15 years

(Paediatric controls - Liverpool only)

Why is this study being done?

This study is looking at a condition called "Lupus". We don't know a lot about what causes it or the best ways to treat it. We hope that by understanding more about what causes Lupus we can create better treatments.

To do this we need to compare children with Lupus to children without Lupus, like you.

Why have I been chosen?

You are being asked to take part in this study because you do not have Lupus.

Do I have to help?

No, it's up to you and your parents or the adult looking after you. You can change your mind at any time during the research without giving a reason. This will not affect the care you receive.

What will happen if I say yes?

- We will take a blood sample (2-3 teaspoons). If you need blood tests for another reason we will take it at the same time.
- We will also ask if you can give us a urine sample.
- We will collect information about your age, gender and your general health.
- We will keep your name and hospital details on a list in the hospitals locked research office so we know you are in the study.
- We will tell your GP you are helping us with the study.

Will it do some good if I say yes?

By doing experiments on your samples, you will be helping us create better treatments for children with Lupus in the future.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and you will not feel any different. Helping us with the study will not affect your hospital care

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Hospital.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What do I have to do now?

If you are happy to take part you will be asked to write your name on a form. This is to say that you understand the study and what will happen. You will be given your own form to keep as well as this leaflet.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

Paediatric control patient information sheet for 16 and over

UK Children's Lupus Study

Information sheet for patients aged 16 years and older

(Paediatric controls – Liverpool only)

What is a study? Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body attacks itself. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with Lupus and without Lupus to see why Lupus cells are reacting against themselves. In addition we need to look at urine to detect inflammation of the kidney, a common complication of Lupus.

This project is part of a UK wide study of children with Lupus.

Why am I being asked to take part?

Because you do not have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect the care you receive.

What will happen if I say yes?

- We will take a sample of your blood (2-3 teaspoons). If you are having blood tests for another reason we will take it at the same time.
- We will also ask if you can give us a urine sample. We use this to look at how the kidneys work in Lupus.
- We will collect information about your age, gender and your general health.
- We will keep your name and hospital details on a list in your hospitals research office so that we know you are in the study. Your participation will be kept strictly confidential.
- We will tell your GP you are helping us with the study

Will it do some good if I say yes?

By doing experiments on the samples given you will be helping us create better treatments for children with Lupus in the future.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and you will not feel any different. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented at scientific conferences and published in medical journals. You will not be identified in any way.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department at Alder Hey, the charity Lupus UK and The Alder Hey Renal Fund.

No one, including your doctor receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric and Research Ethics Committee have approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised. This means that no-one will know it belongs to you.

Forms will be kept in your hospitals research office. They will be stored in locked filing cabinets at all times, in rooms that are locked when non-attended.

All information kept on study computers (in offices of the UK JSLE Study Group at the Institute Child Health, University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this Leaflet!

D9: Paediatric control patient assent form

Alder Hey Children's NHS
NHS Foundation Trust

Assent Form – Controls (on local centre headed paper)

(Paediatric Controls – Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have been told about the study and given the information sheet (Version 4, 1st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand taking part is voluntary and I can stop taking part at any time, without giving any reason, without this making any difference to my medical care or legal rights.	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my blood may be used to investigate Lupus.	
5.	I agree that a small amount of my urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree that I will take part in the above study.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

D10: Paediatric control patient consent form

Alder Hey Children's NHS
NHS Foundation Trust

Patient's Consent Form

(Paediatric Controls - Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 - 1st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my blood may be used to investigate Lupus.	
5.	I agree that a small amount of my urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree that I will take part in the above study.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

D11: Adult volunteer information sheet

UNIVERSITY OF LIVERPOOL

PARTICIPANT INFORMATION SHEET

1. **Study Title:** Analysis of normal blood leukocyte function using cells from healthy volunteers.
2. **Version Number and date.** Version 3, January 2015
3. **Invitation.** You are being invited to participate in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything that you do not understand. Please also feel free to discuss this with your friends, relatives and GP if you wish. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to.
4. **What is the purpose of the study?** The purpose of the study is to derive baseline data for how specialised white blood cells in the blood, called leucocytes, behave in normal healthy donors. These cells normally help prevent infection as part of the normal immune response. This will involve measuring protein expression, cell function and/or analysis of genes related to immune function as part of a series of studies in the Dept of Women's and Children's Health, University of Liverpool.
5. **Why have I been chosen?** You have been chosen because you are a normal healthy person who has indicated they are willing to give blood.
6. **Do I have to take part?** No. It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason.
7. **What will happen if I take part?** You will be asked to give 5-30ml blood. We may ask to take blood again, so that we can reproduce our results, but no more frequently than 10 times per year. However, we will only do this if you are well and completely happy to give the extra samples. You are free to say no and opt out of the study at any stage. Blood samples will be tested in the laboratory at the department of Women's and Children's health. All tests will be performed by the recognised researchers concerned with this project. Samples taken will not be identified by a person's name after collection but will be anonymised and only identified to the research worker by a code number. Samples will be tested immediately not stored in department refrigerators or freezers until tests are performed. Only the investigators will have access to this material. Samples will be destroyed after analysis and will not be used for any other research studies.
8. **Expenses and payments**. We do not offer any payments for participants.

9. **What are the risks in taking part?** There are no risks additional to those normally encountered when giving a blood sample for clinical purposes. You may experience slight bruising at the needle site.

10. **Are there any benefits in taking part?** There are no benefits intended for participants but you will be contributing to research which may eventually lead to the development of improved therapies.

11. **What if i am unhappy or if there is a problem?** If you are unhappy, or if there is a problem, please feel free to let us know by contacting Dr. Brian Flanagan (0151 282 4732) and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide them with name of this study and the researcher(s) involved, and the details of the complaint you wish to make.

12. **Will my taking part in the study be kept confidential?** Yes, the results of the study will be anonymised and any information about you kept confidential. The data may be kept for up to five years on password protected computers.

13. **What will happen to the results of this study?** The results of the research may ultimately be published in scientific papers but you will not be identified in any report.

14. **What will happen if I want to stop taking part?** You are free to withdraw at anytime, without explanation. Results up to the period of withdrawal may be used, if you are happy for this to be done. Otherwise you may request that they are destroyed and no further use is made of them.

15. **Who can I contact if I have further questions?** For any further questions or concerns about participating in this study please contact:

    Dr Brian Flanagan
    Department of Women's and Children's health,
    University of Liverpool,
    Alder hey Hospital,
    Eaton Road,
    Liverpool L12 2AP
    Tel. 0151 282 4732
    email fla1@liv.ac.uk

16. **Duty of care to research participants.** Occasionally research studies can reveal significant unexpected abnormalities which require medical follow-up, either for further investigation or (more rarely) treatment. You will be asked to agree that if any significant abnormality is found, we will send the report to your GP, who will be able to take it further with you. Should this be needed the study PI will discuss this with you.

Thank you for taking the time reading this information sheet, please complete the attached consent form if you wish to participate.

D12: Adult volunteer consent form

UNIVERSITY OF LIVERPOOL

Committee on Research Ethics

PARTICIPANT CONSENT FORM

Title of Research Project: Analysis of normal blood leukocyte function using cells from healthy volunteers.

Researcher(s): Dr BF Flanagan

	Please initial box
1. I confirm that I have read and have understood the information sheet version 3 dated January 2015 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected.	☐
3. Data will be stored electronically in a anonymised form and will not be identified using my name, only a sample code number. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish.	☐
4. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications.	☐
5. If an unexpected significant abnormality is discovered I consent to my GP being contacted	☐
6. I agree to take part in the above study.	☐

Participant Name	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

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APPENDIX E: PUBLICATION (SCIENTIFIC REPORTS)

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The human glomerular endothelial cells are potent pro-inflammatory contributors in an *in vitro* model of lupus nephritis

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Juvenile-onset lupus nephritis (LN) affects up to 80% of juvenile-onset systemic lupus erythematosus patients (JSLE). As the exact role of human renal glomerular endothelial cells (GEnCs) in LN has not been fully elucidated, the aim of this study was to investigate their involvement in LN. Conditionally immortalised human GEnCs (ciGEnCs) were treated with pro-inflammatory cytokines known to be involved in LN pathogenesis and also with LPS. Secretion and surface expression of pro-inflammatory proteins was quantified via ELISA and flow cytometry. NF-κB and STAT-1 activation was investigated via immunofluorescence. Serum samples from JSLE patients and from healthy controls were used to treat ciGEnCs to determine via qRT-PCR potential changes in the mRNA levels of pro-inflammatory genes. Our results identified TNF-α, IL-1β, IL-13, IFN-γ and LPS as robust *in vitro* stimuli of ciGEnCs. Each of them led to significantly increased production of different pro-inflammatory proteins, including; IL-6, IL-10, MCP-1, sVCAM-1, MIP-1α, IP-10, GM-CSF, M-CSF, TNF-α, IFN-γ, VCAM-1, ICAM-1, PD-L1 and ICOS-L. TNF-α and IL-1β were shown to activate NF-κB, whilst IFN-γ activated STAT-1. JSLE patient serum promoted IL-6 and IL-1β mRNA expression. In conclusion, our *in vitro* model provides evidence that human GEnCs play a pivotal role in LN-associated inflammatory process.

Lupus nephritis (LN) is one of the main complications of juvenile-onset systemic lupus erythematosus (JSLE), a rare, multi-system autoimmune disease[1]. LN is a chronic inflammatory renal disease characterised by periodic flares (active LN) and remissions (inactive LN)[2]. Each flare potentially contributes to the accumulation of damage of renal structures[3] such as the glomerulus[4]. LN can affect up to 80% of JSLE patients[5–7], whereas 10–15% of LN patients eventually develop end-stage renal disease[8–12].

The renal glomerulus is part of the nephron, where primary urine formation occurs[13]. Each glomerulus contains a network of glomerular capillaries which, in healthy individuals, effectively filter blood plasma through the glomerular filtration barrier (GFB) restricting blood cells and proteins inside the circulation[13]. In LN, chronic inflammation-induced GFB damage results in proteinuria and haematuria[14,15].

The human glomerular endothelial cells (GEnCs), a component of the GFB, line the glomerular capillary lumen[13]. As the GEnCs directly interact with circulating factors and immune cells in the blood and with the renal glomerular podocytes and mesangial cells, they could be central intra-renal contributors to inflammatory processes. To date, there are limited data concerning human GEnC involvement in LN inflammation and production of pro-inflammatory mediators. However, human umbilical vein endothelial cells (HUVECs)[16,17] and human brain microvascular endothelial cells (HBMECs)[18,19], when cultured *in vitro* under appropriate pro-inflammatory stimuli such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta (IL-1β) and lipopolysaccharide (LPS), have been shown to elicit inflammatory responses. These responses involve nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) activation and production of adhesion molecules and cytokines. Thus, the aim of this study was to investigate the potential inflammatory role of human GEnCs

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in an *in vitro* model of LN. Unravelling their potential involvement in lupus renal disease could provide further insight into LN pathogenesis.

Results

Inflammatory stimulation of ciGEnCs induces the production of urinary biomarkers and pro-inflammatory proteins. The combination of all cytokines (All: 3,755 pg/mL [3,159–3,989], $p = 0.008$) led to statistically significantly higher levels of secreted monocyte chemoattractant protein-1 (MCP-1) compared to the untreated cells (800 pg/mL [346–1,856]) (Fig. 1a), an effect also observed for LPS (2,876 pg/mL [2,457–3,072], $p = 0.04$). A significant increase in secreted levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) (Fig. 1b) was induced only by IL-13 (1,539 pg/mL [731–1,718], $p = 0.022$) compared to the untreated cells (192 pg/ml [74–661]). None of the other novel urinary biomarkers were expressed by ciGEnCs (data not shown).

As the ciGEnCs were shown to mainly produce and secrete only two (MCP-1 and sVCAM-1) out of the seven identified novel urinary biomarkers for LN[20], it was hypothesised that the role of GEnCs may be related to the production and local secretion of other pro-inflammatory mediators. To address this hypothesis, changes in secretion levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6 and IL-10), chemokines (macrophage inflammatory protein-1 alpha (MIP-1α), IFN-γ-induced protein-10 (IP-10) and IL-8) as well as the blood cell growth factors (granulocyte-macrophage and macrophage colony-stimulating factor (GM-CSF and M-CSF)) were examined via Luminex ELISA.

IL-6 secretion was significantly upregulated after IL-1β (3,479 pg/mL [1,599–5,273], $p = 0.0004$) and LPS stimulation (3,323 pg/mL [1,083–5,228], $p = 0.0007$) (Fig. 1c) compared to untreated ciGEnCs (31 pg/ml [11–164]). A trend was observed for increased IL-8 secretion after combined cytokine treatment (All; 6,127 pg/mL [3,664–6,255], $p = 0.066$) compared to untreated cells (623 pg/mL [449–1,503]) (Fig. 1d). Combined cytokine treatment (All: 24 pg/ml [22–24], $p < 0.0001$) and LPS (17 pg/ml [8–21], $p = 0.009$) significantly increased IL-10 secretion compared to untreated cells (0.5 pg/ml [0.3–0.7]) (Fig. 1e). The combined cytokine treatment led to secretion of significantly higher amounts of M-CSF (All: 3,461 pg/ml [946–6,220], $p = 0.02$) compared to untreated cells (192 pg/ml [69–2,077]) (Fig. 1f). IL-1β (2,284 pg/ml [710–2,748], $p = 0.0001$) and TNF-α (522 pg/ml [493–722], $p = 0.02$) significantly increased the secretion of GM-CSF compared to untreated cells (20 pg/ml [17–40]), as did the combined cytokine treatment (All: 1,010 pg/ml [323–1,925], $p = 0.002$) and LPS (790 pg/ml [231–1,882], $p = 0.008$) (Fig. 1g).

TNF-α (222.3 pg/ml [221–224], $p = 0.048$), IL-1β (221.5 pg/ml [214–226], $p = 0.049$) and the combined cytokine treatment (All: 239 pg/ml [233–243], $p = 0.0002$) significantly increased the secretion of MIP-1α compared to untreated cells (209 pg/ml [207–209], as did LPS (226 pg/ml [215–230], $p = 0.011$) (Fig. 1h). IFN-γ (3,015 pg/ml [2,530–3,201], $p = 0.008$) and combination of cytokines (All: 3,222 pg/ml [3,153–3,262], $p = 0.0003$) significantly upregulated IP-10 secretion compared to untreated cells (2 pg/ml [2–5]), as did LPS (2,870 pg/ml [847–3,218], $p = 0.013$) (Fig. 1i).

IFN-γ (8 pg/ml [7–9], $p = 0.029$), IL-1β (11 pg/ml [7–13], $p = 0.011$) and LPS (15 pg/ml [8–21], $p = 0.0005$) treatments significantly upregulated TNF-α secretion compared to untreated cells (4 pg/ml [4–4]) (Fig. 1j). The single TNF-α treatment and the combined cytokine treatment were excluded as they gave false positive results due to the presence of recombinant human TNF-α. LPS treatment (28 pg/ml [2–42], $p = 0.005$) induced the secretion of significantly higher amounts of IFN-γ compared to untreated cells (1 pg/ml [1–1]) (Fig. 1k). The single IFN-γ treatment and the combined cytokine treatment were excluded as they gave false positive results due to the presence of recombinant IFN-γ.

Due to lack of IL-6- and VEGF-induced changes in the mRNA expression levels of MCP-1, VCAM-1, IL-6 and IL-8 compared to untreated ciGEnCs (data not shown), ciGEnC pro-inflammatory protein secretion in IL-6- and VEGF-treated conditioned media was not tested in ELISA assays (the 96-well plate capacity of the Luminex assay also limited the amount of conditioned media tested to those that were expected to exhibit the most meaningful changes in protein secretion compared to the untreated ciGEnCs). However, IL-6 and VEGF were included in the combined cytokine treatments (All).

The combined effect of TNF-α and IL-1β in MCP-1 and M-CSF secretion was also specifically tested. TNF-α together with IL-1β statistically significantly increased MCP-1 secretion (2,561 pg/ml [2,055–3,449], $p = 0.02$) compared to untreated ciGEnCs (1,300 pg/ml [916–2,401]) (Fig. 1l). TNF-α alone significantly increased M-CSF secretion (446 pg/ml [333–975], $p = 0.02$) compared to untreated ciGEnCs (187 pg/ml [136–304]) and combined TNF-α and IL-1β treatment further increased M-CSF secretion (629 pg/ml [446–1,354], $p = 0.001$) (Fig. 1m).

24-hour serum treatments induce changes in mRNA expression levels of pro-inflammatory genes. Treatment of ciGEnCs for 4 h with sera from JSLE- and healthy control (HC) patients did not show any significant differences between groups (data not shown). At 24 h, JSLE serum (0.002 [0.0007–0.003], $p = 0.03$) significantly increased *IL-6* mRNA expression compared to HC (0.001 [0.0006–0.002]) (Fig. 2a). A trend was also observed for increased JSLE sera-induced *IL-1β* mRNA expression (0.003 [0.0008–0.006], $p = 0.07$) compared to HC (0.002 [0.0008–0.004]) (Fig. 2k). Furthermore, a trend was observed for reduced *IP-10* mRNA expression levels after JSLE serum treatments (0.00005 [0.000004–0.005], $p = 0.055$) compared to HC sera (0.0001 [0.00003–0.00080]) with the mRNA in both groups being, in general, lowly expressed (Fig. 2g).

When JSLE serum was categorised as either active (renal BILAG A/B) or inactive LN (renal BILAG D/E), a trend was observed for decreased *IP-10* mRNA levels after inactive LN serum treatments (0.00005 [0.00002–0.0001], $p = 0.06$) compared to HC (0.0001 [0.00003–0.0008]) but no significant difference was demonstrated between HC and active LN serum treatments (Fig. 3g). Similarly, *TNF-α* mRNA expression levels were statistically significantly reduced after inactive LN serum treatments (0.0003 [0.0001–0.000901], $p = 0.04$) but not after active LN serum treatments when compared to HC (0.0008 [0.0004–0.002]) (Fig. 3j). A trend was observed for higher *IL-10* mRNA expression after active LN serum treatments (0.0003 [0.00002–0.003]) compared to the inactive sera (0.00005 [0.00002–0.0001]) although these differences were not statistically significant ($p = 0.08$) (Fig. 3c).

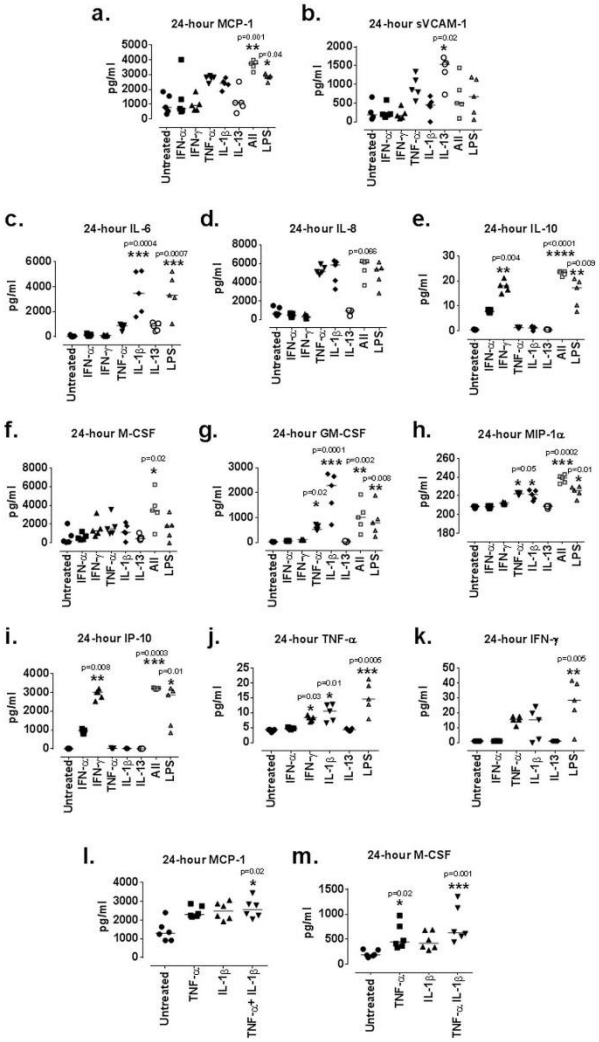

Figure 1. LN urinary biomarker, chemokine, cytokine and growth factor secretion after 24-hour ciGEnC-stimulation with individual cytokines, combination of all cytokines (All) and LPS. MCP-1 (**a**), sVCAM-1 (**b**), IL-6 (**c**), IL-8 (**d**), IL-10 (**e**), M-CSF (**f**), GM-CSF (**g**), MIP-1α (**h**), IP-10 (**i**), TNF-α (**j**) and IFN-γ (**k**) secretion in response to cytokine treatments. MCP-1 (l) and M-CSF secretion (m) following combined TNF-α and IL-1β treatments. N = 5–6/group, Data are presented as median concentrations (pg/ml) [range] are analysed using Kruskal-Wallis test with Dunn's post-hoc test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs untreated.

24-hour serum treatments induce changes in the secretion levels of IL-6. The 24 h JSLE serum treatments were able to promote an increase in the mRNA expression of *IL-1β* and *IL-6*. For this reason, we also investigated the effect of serum treatments in IL-1β and IL-6 secretion by the ciGEnCs. IL-1β and IL-6 in serum samples were also tested to ensure whether potential presence of these cytokines in the ciGEnC conditioned

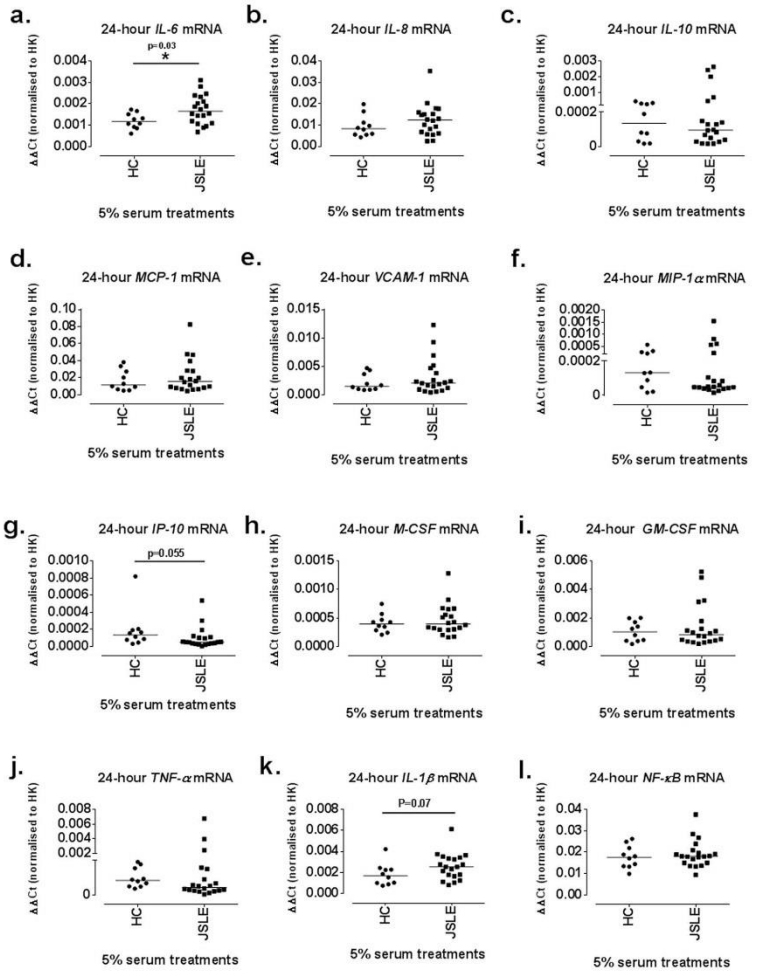

Figure 2. Effect of 24 h ciGEnC treatments with 5% JSLE or HC sera in pro-inflammatory gene mRNA expression. Changes in *IL-6* (**a**), *IL-8* (**b**), *IL-10* (**c**), *M-CSF* (**d**), *GM-CSF* (**e**), *MCP-1* (**f**), *VCAM-1* (**g**), *MIP-1α* (**h**), *IP-10* (**i**), *TNF-α* (**j**), *IL-1β* (**k**) and *NF-κB* (**l**) mRNA expression levels. Data presented as median ΔΔCt [range] are analysed with Mann-Whitney test. *P </= 0.05 vs HC N = 10–20/group.

media could be attributed to ciGEnC production. IL-1β presence in serum samples was relatively low (frequently below the level of detection for the assay) and did not differ among HCs, active and inactive LN patients (Fig. 4a). Furthermore, when ciGEnCs were treated with 5% sera from LN patients, IL-1β levels secreted by the cells were below the level of detection for the assay (except for 1 active disease sera sample) (Fig. 4b).

On the contrary, a trend was observed for higher IL-6 levels in active LN sera (62 pg/ml [21–165], p = 0.054) compared to those of HC sera (13 pg/ml [13–40]) whereas inactive LN sera levels of IL-6 (52 pg/ml [1–267]) were closer to those of active LN sera (Fig. 4c). The active LN (572 pg/ml [390–741], p = 0.01]) and the inactive LN (647 pg/ml [352–792], p = 0.007) serum treatments promoted a statistically significant increase in IL-6 secretion by ciGEnCs compared to untreated ciGEnCs (263 pg/ml [22–314]); the HC serum treatments (471 pg/ml [345–698]) were not able induce as high IL-6 levels as the JSLE sera.

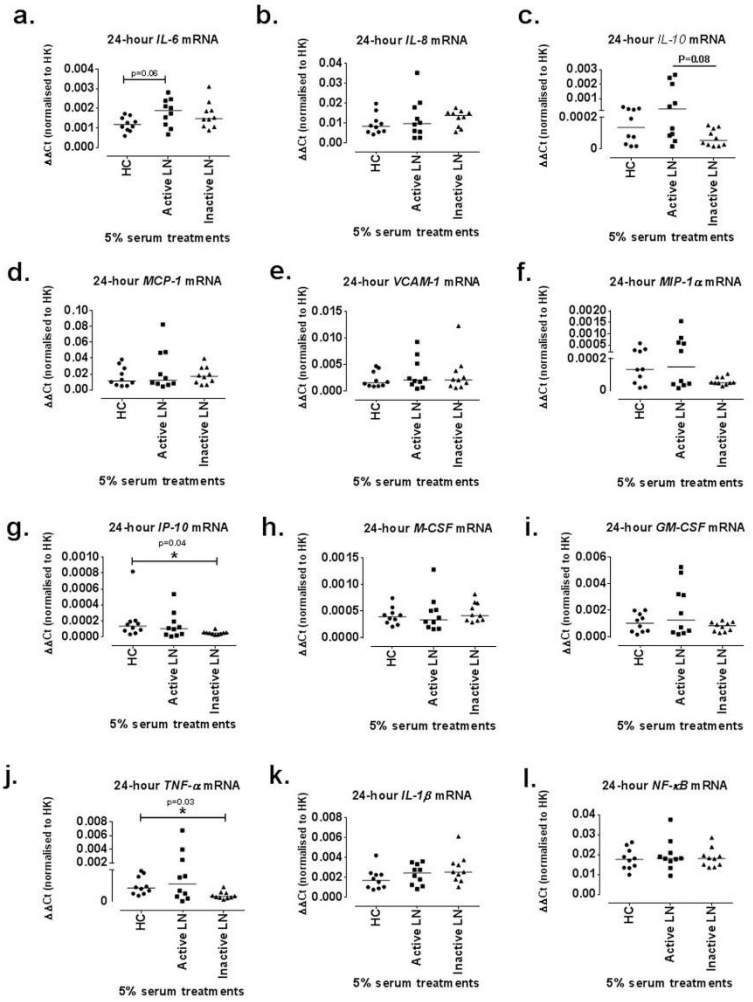

Figure 3. Effect of 24 h ciGEnC treatments with 5% active and inactive LN or HC sera in pro-inflammatory gene mRNA expression. Changes in *IL-6* (**a**), *IL-8* (**b**), *IL-10* (**c**) *M-CSF* (**d**), *GM-CSF* (**e**), *MCP-1* (**f**), *VCAM-1* (**g**), *MIP-1α* (**h**), *IP-10* (**i**), *TNF-α* (**j**), *IL-1β* (**k**) and *NF-κB* (**l**) mRNA expression levels. Data presented as median ΔΔCt [range] are analysed with Kruskal-Wallis with Dunn's post-hoc test. *P </= 0.05. N = 10/group.

Inflammatory protein release is not associated with increased cellular death. The majority of ciGEnCs remained viable (Anx V−/PI−) after 24 h treatment with cytokines and LPS similarly to the untreated ciGEnCs (Supplementary Fig. 1). No significant changes were observed in the levels of apoptotic (Anx V+/PI−) and late apoptotic/necrotic (Anx V+/PI+) ciGEnCs among the different treatments (Supplementary Fig. 1).

Inflammatory mediators induce cell adhesion and co-stimulatory molecule expression in ciGEnCs. The endothelium is responsible for the recruitment and activation of leukocytes to the glomerular

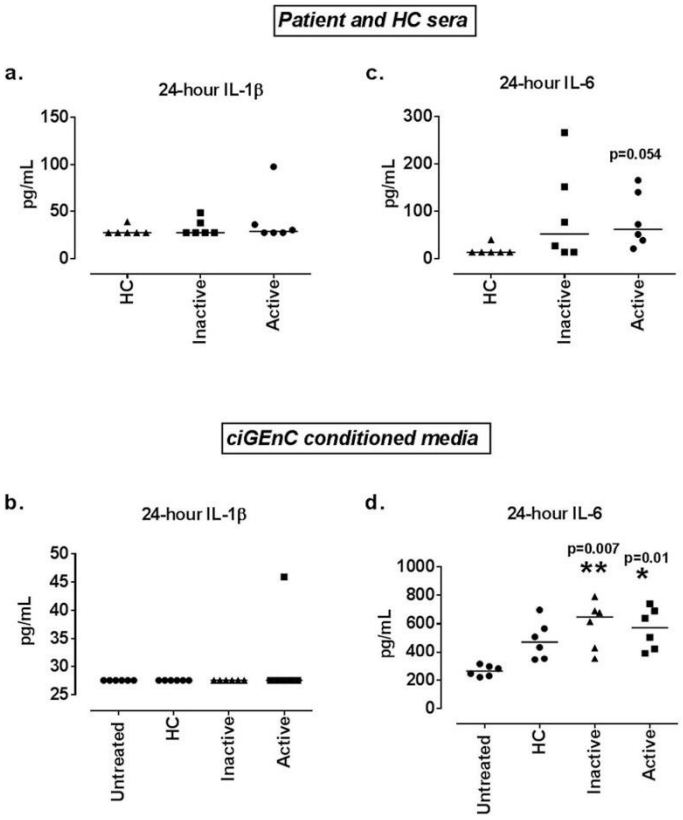

Figure 4. Effect of 24 h ciGEnC treatments with 5% active and inactive LN or HC sera in IL-1β and IL-6 secretion. IL-1β levels in active and inactive LN patient and HC sera **(a)** and IL-1β levels in ciGEnC conditioned media **(b)**. IL-6 levels in active and inactive LN patient and HC sera **(c)** and IL-6 levels in ciGEnC conditioned media **(d)**. Data presented as median concentrations (pg/ml) [range] are analysed using Kruskal-Wallis test with Dunn's post-hoc test. *P </= 0.05, **P < 0.01 vs HC or vs untreated ciGEnC conditioned media. N = 6/group.

space by upregulating expression of cellular adhesion and co-stimulatory molecules. The role of TNF-α, IL-1β, IL-13, IFN-γ and LPS was therefore assessed in modulating VCAM-1, intercellular adhesion molecule-1 (ICAM-1), programmed death-ligand 1 (PD-L1) and inducible co-stimulator-ligand (ICOS-L) surface expression.

TNF-α and IL-13, alone or combined, had no significant effect on VCAM-1 and ICAM-1 surface expression at 4 h (data not shown) in contrast to 24 h, where TNF-α (15 [7–86], p = 0.008) and more robustly the combination of IL-13 and TNF-α (54 [11–157], p = 0.0007) significantly upregulated surface VCAM-1 compared to untreated ciGEnCs (3 [0–5]) (Fig. 5a). At 24 h, TNF-α significantly upregulated surface expression of the constitutively expressed ICAM-1 (19,789 [13,348–25,897], p = 0.009) (Fig. 5c) compared to untreated cells (6,609 [3,984–9,632]) but IL-13 had no effect on ICAM-1. IL-1β and LPS treatments had an effect predominantly on ICAM-1. IL-1β and LPS significantly increased surface ICAM-1 at 4 h (data not shown) compared to untreated cells, and this effect was sustained and further enhanced after 24 h (IL-1β: (6,562 [5,953–9,673], p = 0.004), LPS: (7,997 [5,497–12,334], p = 0.002) compared to untreated cells (1,727 [1,339–3,161) (Fig. 5d). No surface VCAM-1 upregulation by IL-1β, IFN-γ and LPS was observed at 24 h (untreated ciGEnC measurements were lower than those of the isotype control and therefore have been assigned 0-values) (Fig. 5b). E-/P-selectin surface expression was not modified by cytokine treatments (Supplementary Fig. 2). IFN-γ had no prominent effect on ICAM-1 or VCAM-1 but significantly increased the surface expression of PD-L1 (IFN-γ: 3,549 [2,199–3,828], p = 0.02), a negative T-cell co-stimulatory molecule[21], after 24 h compared to untreated ciGEnCs (1,763

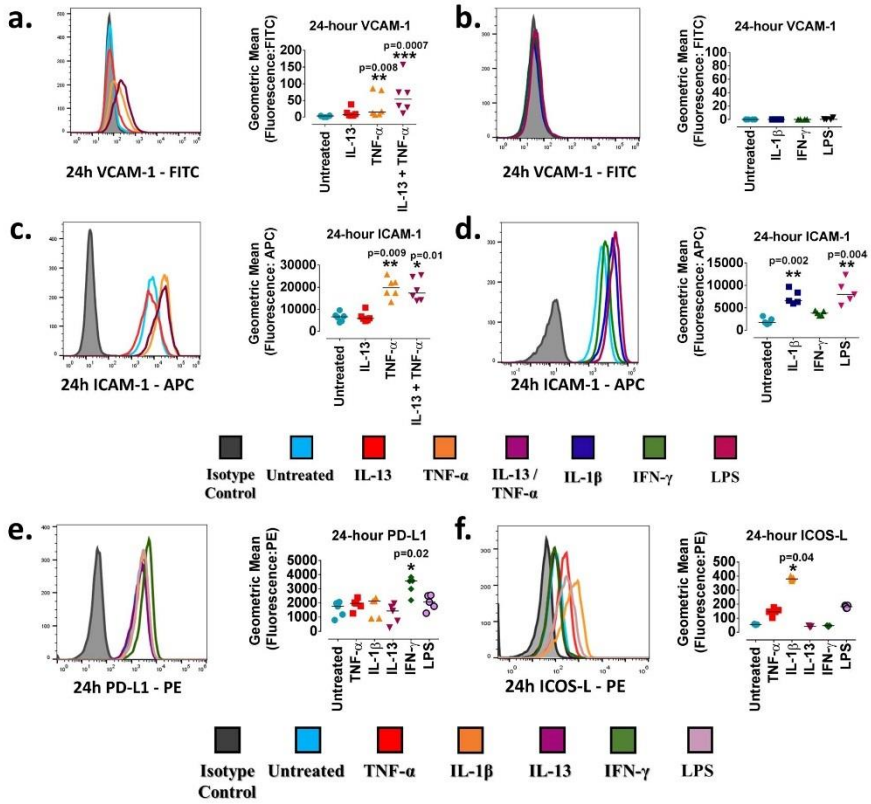

Figure 5. VCAM-1, ICAM-1, PD-L1 and ICOS-L surface expression following 24-hour stimulation with cytokines and LPS. (**a**) 24-hour VCAM-1 surface expression following IL-13 and TNF-α treatments. (**b**) 24-hour VCAM-1 surface expression following IL-1β, IFN-γ and LPS treatments. (**c**) 24-hour ICAM-1 surface expression following IL-13 and TNF-α treatments. (**d**) 24-hour ICAM-1 surface expression IL-1β, IFN-γ and LPS treatments. (**e**) 24-hour PD-L1 surface expression following TNF-α, IL-1β, IL-13, IFN-γ and LPS treatments. (**f**) 24-hour ICOS-L surface expression following TNF-α, IL-1β, IL-13, IFN-γ and LPS treatments. N = 4–6/group. Data presented as median [range] are analysed using Kruskal-Wallis test with Dunn's post-hoc test. *P </= 0.05, **P < 0.01, ***P < 0.001. N = 4–6/group.

[793–2,067]) (Fig. 5e). Finally, surface ICOS-L expression was significantly increased only at 24 h by IL-1β (380 [365–401], p = 0.04) compared to untreated ciGEnCs (55 [52–59]) (Fig. 5f).

TNF-α promotes neutrophil adhesion to ciGEnCs. Adhesion of human neutrophils on ciGEnCs was tested following 24 h incubation of ciGEnCs with TNF-α and IL-13 alone, or in combination. Following 24 h incubation, the numbers of neutrophils bound on the ciGEnCs were counted (Fig. 6a–d). TNF-α ciGEnC treatment promoted the adhesion of a statistically significantly higher number of neutrophils ([90 cells [71–142], p = 0.027) (Fig. 6b) compared to untreated ciGEnCs (52 cells [34–60]) (Fig. 6a, 6e). The effect of the combined TNF-α and IL-13 treatments did not differ from that of TNF-α alone and led to ciGEnC adhesion of a statistically significantly higher number of neutrophils ([99 cells [69–151], p = 0.03), compared to the untreated ciGEnCs (52 cells [34–60]) (Fig. 6d, 6e). Treatments of ciGEnCs with IL-13 [46 cells [23–61]) did not increase neutrophil adhesion compared to untreated ciGEnCs (52 cells [34–60]) (Fig. 6c, 6e).

Pro-inflammatory protein release is occurring through NF-κB and STAT1 induction in ciGEnCs. In order to determine whether the effects observed in ciGEnCs following cytokine and LPS treatments are

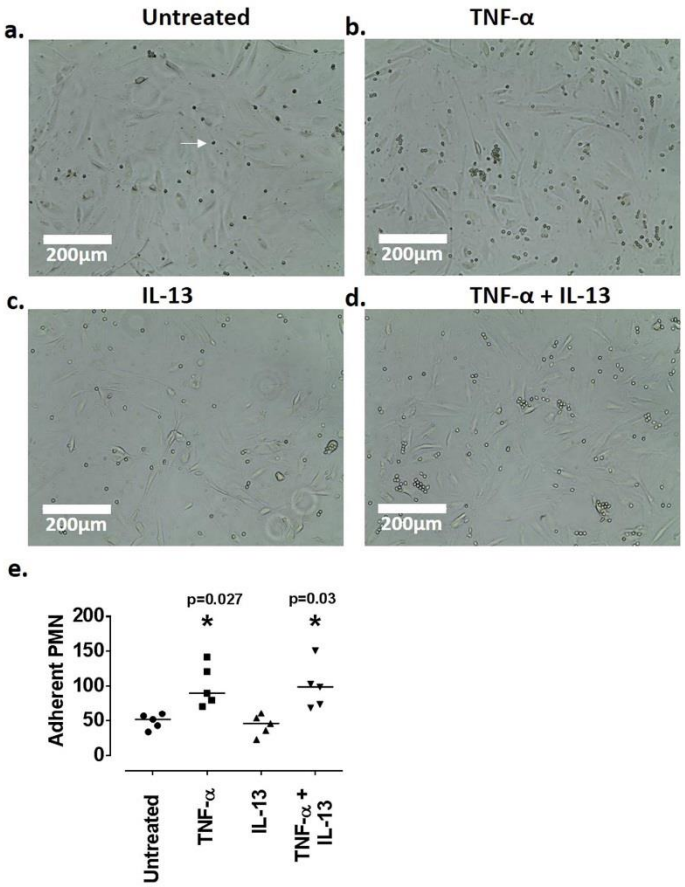

Figure 6. Neutrophil adhesion assay following 24 h stimulation of ciGEnCs with TNF-α and IL-13. (**a**) Neutrophil adhesion on untreated ciGEnCs. (**b**) Neutrophil adhesion on TNF-α-treated ciGEnCs. (**c**) Neutrophil adhesion on IL-13-treated ciGEnCs. (**d**) Neutrophil adhesion on TNF-α + IL-13-treated ciGEnCs. White arrow on (**a**) indicates neutrophil. (**e**) Graph of statistical analysis of neutrophil adhesion assay. Data presented as median cell number [range] are analysed using Kruskal-Wallis test with Dunn's post-hoc test. *P </= 0.05 vs untreated. N = 6/group. Scale bars: 200 µm.

mediated through the NF-kB pathway, we initially used an immunoblot assay to determine nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Iκ-Bα) degradation, as Iκ-Bα is the cytoplasmic inhibitor of NF-κB that prevents its nuclear translocation[22]. At 30 min, it was mainly IL-1β and, to a lesser extent, TNF-α, which were able to induce Iκ-Bα degradation (Fig. 7a).

Nuclear translocation of NF-kB and of signal transducer and activator of transcription (STAT)-1 and -2 (STAT-1 and STAT-2) was tested via immunofluorescence to determine pathway activation. TNF-α and IL-1β promoted NF-κB nuclear translocation at 30 min with IL-1β displaying a more robust effect than TNF-α, whereas LPS only induced low levels of NF-κB activation (Fig. 7b). IFN-γ induced STAT-1 nuclear translocation at 30 min (Fig. 7c) while it induced only moderate to low STAT-2 activation (Fig. 7d).

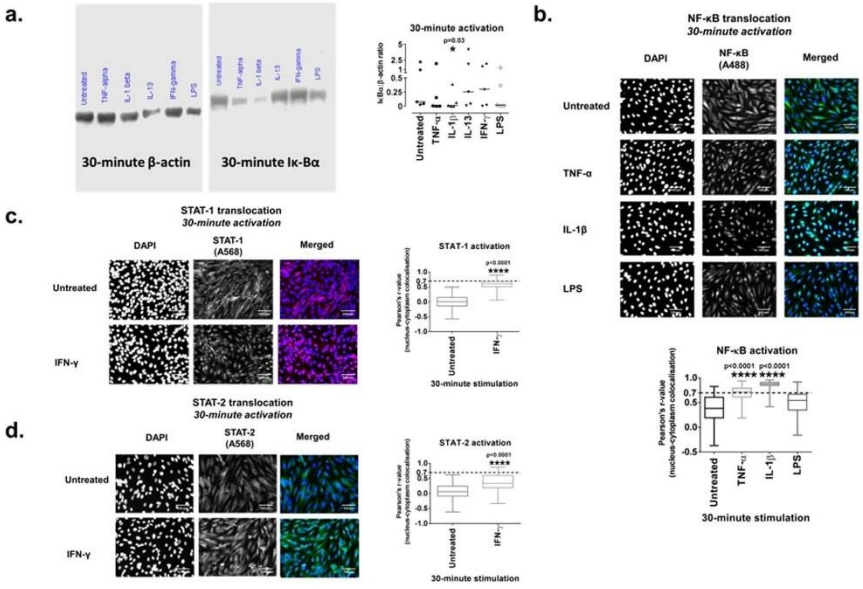

Figure 7. Western blotting for Iκ-Bα protein expression, 20x immunofluorescence images of ciGEnCs and diagrams of statistical analysis for NF-κB, STAT-1 and STAT-2 activation and nuclear translocation, after 30 minutes of stimulation with cytokines and LPS. (**a**) Representative image and graph of Iκ-Bα and beta-actin Western blots (blots have been cropped), following 30-minute stimulation. Data presented as (Mean +/− SEM) in ratio diagrams are analysed using Friedman test with Dunn's post-hoc test, *$P < 0.05$ vs untreated. (**b**) Treatments of ciGEnCs with TNF-α, IL-1β and LPS for 30 minutes for NF-κB nuclear translocation. (**c**) Treatments of ciGEnCs with IFN-γ for 30 minutes for STAT-1 nuclear translocation. (**d**) Treatments of ciGEnCs with IFN-γ for 30 minutes for STAT-2 nuclear translocation. Data (r-values for nuclear co-localisation of DAPI and A488 or A568 for each treatment group) representative of three similar experimental repeats are presented as box and whisker plots and are analysed using Kruskal-Wallis test with Dunn's post-hoc test. ****$P < 0.0001$ vs untreated. Scale bars: 100 μm.

Discussion

LN is one of the main and most significant complications of JSLE. LN affects the majority of JSLE patients (approx. 80%)[5–7], and can cause severe renal damage[3] and GFB impairment[4]. This study aimed to investigate the potential role of the human glomerular endothelium in LN inflammation using a cytokine-based *in vitro* model of LN.

The ciGEnCs were treated with cytokines previously found by our laboratory and by other studies to be elevated in plasma and/or serum samples of JSLE patients with LN (IFN-α[23], IFN-γ[24], TNF-α[24,25], IL-1β[24], IL-6[24], IL-13[24] and VEGF[24,26]) or shown by other studies to be expressed in the glomerular renal tissue of JSLE or adult patients with LN (IFN-γ[27]), or to have certain polymorphisms associated with JSLE (IL-1β[27,28]). The potential effect of bacterial endotoxin inflammation was also examined via 1 μg/ml LPS treatment, which, in murine models of LN and in human endotoxemia, is known to play a critical role in LN exacerbation after a bacterial infection[28–31].

Our findings indicate that in line with *in vitro* models using HUVECs[17,32,33] and HBMECs[34–36], upon activation with TNF-α, IL-1β, IL-13, IFN-γ and with LPS, ciGEnCs significantly increase the production of key pro-inflammatory proteins. These included: cytokines, chemokines, blood cell growth factors, adhesion molecules and T-cell co-stimulatory molecules. All proteins demonstrated in this study to be upregulated in human ciGEnCs have been previously shown to be involved in human LN or other types of human renal disease and are summarised on Table 1.

Single TNF-α and IL-1β treatments upregulated MIP-1α and GM-CSF and combined TNF-α and IL-1β treatment significantly increased MCP-1 secretion. LPS also had a prominent effect in MIP-1α and GM-CSF secretion. MCP-1 promotes infiltration by inflammatory dendritic cells in LN[37] and MIP-1α can activate and attract human granulocytes, macrophages and monocytes[38] whereas GM-CSF promotes the maturation and differentiation of macrophages, neutrophils, eosinophils and basophils[39]. IL-1β and LPS also upregulated IL-6 and TNF-α

Proteins induced by TNF-α, IL-1β, IL-13, IFN-γ and LPS	Involvement in SLE and renal disease
MCP-1	MCP-1 expression within the glomerulus can be predictive of poor outcomes in JSLE patients with LN[37].
VCAM-1	VCAM-1 serum and urinary levels have been shown to be upregulated and to be associated with active LN[69,70].
ICAM-1	ICAM-1 urinary levels are upregulated in patients with class III, IV and V LN[71].
IL-6	High IL-6 levels have been detected in the urine of active LN patients[72].
IL-8	High IL-8 levels have been detected in the urine of active LN patients[72].
IL-10	Plasma IL-10 has been found to be significantly elevated in JSLE patients with active disease[73].
M-CSF	Serum and urine M-CSF levels have been shown to be a reliable and sensitive biomarkers for LN[74,75].
GM-CSF	Increased mRNA levels of GM-CSF are expressed by *in vitro* cultured human renal tubular cells derived from interstitial fibrotic kidneys compared to tubular cells derived from non-fibrotic kidneys[76].
MIP-1α	Together with MCP-1, MIP-1α promotes macrophage recruitment and activation in the kidneys of patients with crescentic GN[77].
IP-10	IP-10 serum levels are upregulated in LN patients[78].
TNF-α	TNF-α gene polymorphisms leading to excessive serum TNF-α production can predispose patients to LN development[79].
IFN-γ	Significantly increased IFN-γ positive immune-histochemical staining has been observed in biopsies from juvenile-onset LN patients compared to HCs[27].
PD-L1	In JSLE, decreased PD-L1 expression by antigen-presenting cells has been associated with active disease[80].
ICOS-L	ICOS-L plasma levels are increased in patients with active SLE compared to those with inactive SLE[81].

Table 1. Proteins produced by activated ciGEnCs and their involvement in renal disease.

secretion. IL-6 promotes B- and T-cell differentiation[40] whereas TNF-α is a prototypical pro-inflammatory cytokine[41] affecting a broad variety of cell types, including the human endothelium.

IFN-γ increased the secretion of IP-10, IL-10 and TNF-α, as did LPS. IP-10 mediates T-cell accumulation to the inflamed tissues[42] whereas IL-10 is involved in autoantibody production[43]. The combined cytokine treatment, which could be mainly attributed to TNF-α, IL-1β and IFN-γ led to increased amounts of secreted M-CSF as the other cytokines had minimal effects individually. When the combined effect of the prototypical pro-inflammatory cytokines -TNF-α and IL-1β- was specifically tested, the ciGEnCs were found to significantly upregulate M-CSF secretion compared to the untreated ciGEnCs. M-CSF promotes the proliferation, differentiation, and survival of monocytes and macrophages[44]. LPS had a prominent effect on secretion of IFN-γ which is involved in anti-viral immunity[45]. TNF-α and IL-1β appeared to increase secretion of IL-8, a potent neutrophil chemokine[46] although not to a level of statistical significance tested. These data suggest that GEnCs secrete mediators into the environment that induce the recruitment, activation and maturation of inflammatory leukocytes leading to perpetuation of the inflammatory response.

Two important adhesion molecules were found to be upregulated by the ciGEnCs following pro-inflammatory stimulation; VCAM-1 and ICAM-1. VCAM-1 is involved in the binding of lymphocytes, monocytes, eosinophils, and basophils but not neutrophils to the vascular endothelium[47] whereas ICAM-1 promotes all leukocytes' binding to the endothelium[48]. In this study, IL-13 had an effect exclusively on soluble and surface VCAM-1 expression with the latter being promoted by IL-13 in combination with TNF-α. ICAM-1 expression was significantly increased by TNF-α, IL-1β and LPS. 24 h ICAM-1 upregulation by TNF-α, IL-1β and LPS has also been confirmed in HUVECs[49,50]. Similar to the human ciGEnCs and in contrast to other types of human endothelial cells (carotid, coronary), lack of 24 h IL-1β- and LPS-mediated upregulation of surface VCAM-1 has been previously observed in HUVECs and human subclavian endothelial cells[50].

The differential cytokine regulation of surface VCAM-1 and ICAM-1 expression could imply that certain pro-inflammatory stimuli could lead to preferential intra-renal accumulation of certain immune cell populations. Indeed, our findings from the neutrophil adhesion assay indicated that combinatorial TNF-α and IL-13 treatment of ciGEnCs had an effect similar to that of TNF-α alone and led to ciGEnC adhesion of similar numbers of neutrophils, which were significantly higher than those adhering to untreated or IL-13-treated ciGEnCs. Thus, ICAM-1 that was found in this study to be induced by TNF-α but not IL-13 (IL-13 had an effect only on VCAM-1 expression), could be the adhesion molecule predominantly responsible for neutrophil binding on ciGEnCs. This assumption is further corroborated by evidence in the literature that, in contrast to VCAM-1, ICAM-1 can promote binding of neutrophils on the endothelium[48].

In this study an attempt was made to assess the expression of E/P-selectin by the ciGEnCs in response to our pro-inflammatory model however no expression was seen. A recent study demonstrated that GEnCs do not require E/P-selectin for neutrophil binding due to an increased dwell time by cells at this location[51]. This has been confirmed both in mouse models using lupus-prone mice and in human renal biopsies from 108 patients with primary renal disease and allograft recipients[52–55].

PD-L1 surface expression that was upregulated at 24 h in ciGEnCs by IFN-γ treatment, can prevent the activation of $CD\text{-}8^+$ T-cells[21] and inhibit the autoreactive T-cell function[56]. However, after 24 h, IL-1β significantly increased surface expression of the positive $CD4^+$ T-cell co-stimulatory molecule, ICOS-L[57]. Therefore, T-cell-driven kidney inflammation could be promoted by $CD4^-$ instead of $CD8^+$ T-cells.

Incubation of ciGEnCs for 4 h and 24 h reflects acute phase inflammation[58] rather than chronic inflammation. The 24 h cytokine and LPS stimulation did not significantly induce apoptotic or necrotic cell death, indicating that during acute inflammation human ciGEnCs maintain their integrity and acquire a pro-inflammatory phenotype

due to cell activation and not due to cell death. Downstream activation of the central pro-inflammatory transcription factor NF-κB primarily occurred following IL-1β and TNF-α stimulation and to a lesser extent by LPS and was demonstrated by nuclear NF-κB translocation as well as by Iκ-Bα degradation. In contrast, IFN-γ was found to activate downstream STAT-1 signalling but not STAT-2 which is normally activated by IFN-α.

NF-κB shRNA knock-down in human microvascular endothelial cells (HMECs) has been shown to lead to strong suppression of the expression of TNF-α-induced genes such as *MCP-1*, *M-CSF*, *GM-CSF*, *IL-6* and *IL-8*[59]. Furthermore, STAT-1 siRNA knock-down in IFN-γ-treated HUVECs has been shown to downregulate secretion of IP-10 and other T-cell related chemokines[60] providing supporting evidence that these pathways are activated in endothelial cells. Thus, it could be hypothesised that in human GEnCs similar mechanisms of action could be occurring.

The 24 h serum treatments did not demonstrate significant changes in the mRNA expression levels of most pro-inflammatory genes tested. However, *IL-6* and *IL-1β* mRNA levels were increased by the JSLE sera. In addition, IL-6 secretion by the ciGEnCs underwent a pronounced increase after both active and inactive LN serum treatments. On the contrary, IL-1β secretion by ciGEnCs was not affected by either of the serum treatments. This finding suggests potential lack of inflammasome and caspase-1 activation in ciGEnCs following serum treatments which could lead to a reduction in the release of active IL-1β within the microenvironment.

The limited amount of blood samples due to rarity of the disease as well as the small blood sample volumes obtained from paediatric patients did not allow for further investigation and detection of the JSLE serum factors inducing the expression IL-6 and IL-1β production. A potential candidate, however, could be autoantibodies present in the sera samples. Indeed, previous research studies using affinity purified IgG antibodies from anti-endothelial cell antibody (AECA)-positive SLE patients to treat HUVECs *in vitro*, demonstrated increased HUVEC-derived secretion of IL-1[61] and IL-6[62]. Similar findings have also been observed in scleroderma and systemic vasculitis patients[62,63].

The lack of strong responses induced by the JSLE sera may be due to the immunosuppressant regimens followed by patients. Increasing the serum doses used in these experiments may be required to demonstrate an effect; however, the 5% treatments (limited due to sample volumes from paediatric patients) did not suffice to induce a strong *in vitro* ciGEnC pro-inflammatory response.

Furthermore, the serum treatments could have had a more pronounced effect on ciGEnCs in an *in vitro* model in which ciGEnCs would be co-cultured with human podocytes and/or mesangial cells. Lack of crosstalk between native glomerular renal cells could be affecting the results obtained from the serum treatments. The development of a co-culture model, however, was beyond the scope of this study, the aim of which was to investigate pro-inflammatory properties of the human ciGEnCs, a type of human endothelial cells that has been little studied.

There are inherent limitations with using a cell line to generate a model of human disease. However, ciGEnCs retain all the morphological and functional characteristics of the primary GEnCs and express at levels comparable to those of primary GEnCs, the majority of endothelial cell-specific markers[64]. These characteristics render the ciGEnCs into a reliable tool for the study of human GEnCs. Future work could focus on validating these findings using primary cells or in an *in vivo* murine model however this was beyond the scope of this study.

In conclusion, our data imply that the glomerular endothelium could have a pivotal role in LN inflammation (Fig. 8). As demonstrated by our *in vitro* model, the GEnCs are not just passive bystanders but actively respond to the renal pro-inflammatory environment created in kidney disease.

Materials and Methods

Materials. Recombinant cytokines were purchased from Peprotech, LPS was purchased from Sigma. All antibodies are listed on Supplementary Table 1.

Methods. All methods reported here were carried out in accordance with relevant guidelines and regulations of the University of Liverpool. All experimental protocols were reviewed and approved by either the North West – Liverpool East Research Ethics Committee (UK JSLE Cohort Study and Repository) (REC: 06/Q1502/77) or the University of Liverpool Committee of Research Ethics (Adult Healthy Control samples) (Research Ethics Approval Number: RETH000773). Informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian.

Human conditionally immortalised glomerular cell line culture. The ciGEnCs were developed and kindly donated by Professor Moin Saleem and Dr Simon Satchell (Children's Renal Unit, Bristol Royal Hospital for Children)[64]. The ciGEnCs were cultured at 33 °C (with 5% CO_2) in endothelial growth medium 2-microvascular (EGM-2 MV Bulletkit, CC-3202, Lonza) containing 5% Fetal Bovine Serum (FBS) and growth factors as supplied (VEGF was excluded as it has been utilised for treatments). Once cells reached 50–70% confluence they were thermoswitched at 37 °C (with 5% CO_2) for seven days until complete differentiation was achieved and confirmed via light microscopy.

For all experiments, a minimum of three consecutive ciGEnC passages was used for reproducibility purposes. Consecutive passages between 29 and 35 were used, as the researchers who developed and donated the ciGEnCs had previously shown that ciGEnCs can retain their primary GEnC-specific morphological and functional characteristics up to passage 41[64]. All changes in mRNA expression or protein expression, secretion and production levels were compared to those of the untreated ciGEnCs. The fully differentiated ciGEnCs were incubated at 37 °C for 30 mins, 4 h and 24 h with 10 ng/ml (a concentration previously used to induce pro-inflammatory changes in endothelial cells *in vitro*[65–67]) - of seven human recombinant cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-13, VEGF), individually and in combination (All) (cytokine concentration response assays were also performed and are presented on Supplementary Fig. 4) and with 1 μg/ml of LPS.

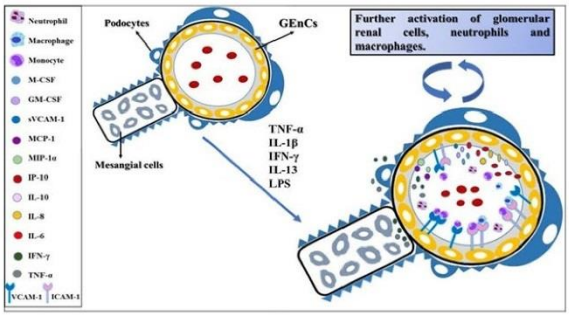

Figure 8. Potential effect of stimulated GEnCs in LN based on the *in vitro* findings. Under the highly inflammatory environment created within the glomeruli by the presence of TNF-α, IL-1β, IL-13 and IFN-γ but also by the potential presence of LPS, the human GEnCs will be activated to produce, secrete and express a variety of pro-inflammatory cytokines, chemokines, blood cell growth factors and adhesion molecules that will further exacerbate the renal disease by either promoting the infiltration of immune cells within the glomeruli or by activating their neighbouring renal cells, the podocytes and mesangial cells.

Fully differentiated ciGEnCs were treated for 4 h or 24 h with 5% (5% sera concentration matching the 5% FBS concentration in standard media used for ciGEnC culture) serum samples in medium without FBS, from JSLE patients with active LN (renal BILAG: A/B, N = 10) and inactive LN (renal BILAG: D/E, N = 10) and with 5% sera from age-matched healthy controls (N = 10). Following completion of treatments, qRT-PCR was performed as above to assess changes in the mRNA expression levels between ciGEnC groups treated with HC and LN sera. Patient and HC information are presented on Table 2. To test the effect of serum samples on IL-6 and IL-1β secretion by the ciGEnCs, an additional of N = 6 of active LN, inactive LN and HC serum samples were collected and used to treat the ciGEnCs for 24 h, at a 5% concentration (patient and HC information presented on Table 3).

qRT-PCR. RNA was extracted using Trizol-chloroform and in-column DNase digestion (Qiagen RNeasy Mini Kit). RNA concentrations and purity were determined using Nanodrop (ND-1000 Spectrophotometer, Thermo Fisher Scientific). 200 ng RNA was transcribed into cDNA using the AffinityScript multi-temp cDNA synthesis kit (Agilent Technologies, Cheshire, UK) as per manufacturer's instructions. qRT-PCR was performed using primers described in Supplementary Table 2 with the Brilliant III Ultra-fast SYBR QPCR mastermix kit (Agilent Technologies) as per manufacturer's instructions. mRNA expression for target genes was normalised using the mean (for one housekeeping (HK) gene) or the geometric mean (for more than one HK genes) of the following HK genes: beta-actin (ACTB), TATA-binding protein (TBP), beta-tubulin (TUBB). The ΔΔCt value was calculated as follows:

$$\Delta\Delta Ct = 2^{-(\textbf{Mean Ct}\,[\text{target gene}] - (\textbf{Geo})\textbf{Mean Ct}\,[\text{HK gene}(s)])}.$$

ELISA assays. The secretion of MCP-1, sVCAM-1, IL-6, IL-8, IL-10, M-CSF, GM-CSF, MIP-1α, IP-10, TNF-α and IFN-γ was tested with single ELISAs (R&D Systems DuoSets) or a Luminex multiplex ELISA assay, utilising conditioned media from ciGEnCs treated for 24 hours with cytokines (as above) and LPS. Conditioned media from IL-6 and VEGF treatments were excluded as they induced no change in the mRNA expression of the urinary biomarkers. However, IL-6 and VEGF were still included in the combined cytokine treatment (All).

Flow cytometry. For VCAM-1, ICAM-1, E-/P-selectin, PD-L1 and ICOS-L detection, the ciGEnCs were stimulated for 24 and then were stained for 30 min on ice in the dark with fluorescence-conjugated antibodies against VCAM-1-FITC, ICAM-1-APC, E-/P-selectin-PE, PD-L1-PE and ICOS-L-PE with appropriate isotype controls. Apoptosis was assessed using the Annexin V/PI kit (Sigma) as per manufactureur's instructions. Flow cytometry was performed using a Merck Guava Flow Cytometer and data were analysed using FlowJo 10.3 software.

Western blotting (WB). For the WB experiments, ciGEnCs were stimulated for 30 min, proteins were extracted using RIPA buffer and protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). 15 μg of protein was diluted in NuPAGE LDS Sample buffer (4x, Invitrogen) at a final 1x concentration with 50 μM dithiothreitol (DTT, Sigma-Aldrich). The protein samples were loaded into 50 μl 12% 10-well precast polyacrylamide gels and transferred to PVDF membranes (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Antibodies were used for the detection of human beta-actin (β-actin) and Iκ-Bα. Following gel transfer, membranes were blocked in TBS-T with 5% milk for 1 h, were incubated with the primary antibodies overnight at 4 °C, washed in TBS-T and incubated with secondary antibodies

Demographics	Active LN (10)	Inactive LN (10)	Healthy Controls (10)
Age (years) (median [range])	15 [9–17]	14.5 [11–18]	13 [9–17.2]
Age at diagnosis (years) (median [range])	12.4 [6.28–14.48]	11.73 [9.3–15.59]	—
Females (%)	90 (9)	90 (9)	70 (7)
Nationality (%)	0	0	90 (9)
White British	10 (1)	0	0
Black British	10 (1)	10 (1)	0
British	0	30 (3)	0
Indian	0	10 (1)	0
Nepalese	10 (1)	30 (3)	0
Pakistani	10 (1)	0	0
Bangladeshi	10 (1)	0	0
Chinese	40 (4)	10 (1)	0
African	0	10 (1)	0
Caucasian	10 (1)	0	10 (1)
Not stated			
Renal BILAG score (%)	30% (3)	80% (8)	—
A	70% (5)	20% (2)	
B			
D			
E			
Renal disease manifestations (%)	70 (7)	—	—
Proteinuria (ACR > 100 mg/mmol)	10 (1)	—	
Nephrotic Syndrome	10 (1)	—	
Renal Hypertension	10 (1)	—	
Low GFR (<80 ml/min/1.73 m^2)	10 (1)	—	
Increased plasma creatinine (>130 μmol/ml)	10 (1)	—	
Urine ACR (mg/dL) (median [range])	—	80 (8)	
Previous renal involvement			
Biopsy-proven LN (%)	70 (7)	30 (3)	—
LN class (WHO or ISN/RPS) (%)	10 (1)	—	
II	10 (1)	—	
III/IV	—	30 (3)	
IV	10 (1)	—	
IV-C	20 (2)	—	
V	10 (1)	—	
IV/V	10 (1)	—	
Mixed			
Medications dosage [range] (patient n-number)	200 mg [200–700] (7)	200 mg [200–300] (7)	—
Hydroxychloroquine	150 mg [100–300] (3)	100 mg [100–100] (2)	
Azathioprine	1500 mg [1200–2000] (5)	1500 mg [750–2000] (6)	
Mycophenolate mofetil	7.5 mg [2.5–45] (6)	10 mg [2–10] (6)	
Prednisolone	—	20 mg [17.5–22.5] (2)	
Methotrexate (oral)	2000 mg (1), 6 pulses (1)	—	
Rituximab	520 mg/m^2 (1)	—	
Cyclophosphamide	—	2 g (1)	
IVIG			

Table 2. Serum treatments for changes in ciGEnC pro-inflammatory gene expression. Age, gender, ethnicity, renal BILAG scores and medications for JSLE patients; age, gender and ethnicity for paediatric healthy controls (N = 10/group).

for 1 h (room temperature). Protein detection was achieved using enhanced chemiluminescence (ECL) reagents (Li-COR). Li-COR software was used for protein band densitometry. Due to β-actin and Iκ-Bα having a similar molecular weight, the samples were loaded and were run twice onto two different gels, which were blocked for either β-actin or Iκ-Bα (full-length images are presented on Supplementary Fig. 3).

Neutrophil adhesion assay. ciGEnCs were stimulated with 10 ng/ml of TNF-α and IL-13, alone or in combination, for 24 h (untreated ciGEnCs were included). Whole-blood from adult healthy controls was used to isolate human neutrophils via HetaSep (StemCell)-aided separation of white blood cells from red blood cells

Demographics	Active LN (6)	Inactive LN (6)	Healthy Controls (6)
Age (years) (median [range])	15.53 [12.18–16.58]	14.55 [11.03–17.79]	14.9 [12.12–16.6]
Age at diagnosis (years) (median [range])	12.8 [6.28–13.29]	10.46 [6.28–16.88]	—
Females (%)	100 (6)	100 (6)	100 (6)
Nationality (%)	16.6 (1)	33.3 (2)	100 (6)
White British	16.6 (1)	16.6 (1)	0
Chinese	33.3 (2)	33.3 (2)	0
Somali	16.6 (1)	16.6 (1)	0
African	16.6 (1)	0	0
Indian			
Renal BILAG score (%)	16.7% (1)	66.7% (4)	—
A	83.3% (5)	33.3% (2)	
B			
D			
E			
Renal disease manifestations (%)	16.6 (1)	0	—
Renal Hypertension (%)	239.3 [0.7–592.4]	7.5 [0.8–8.8]	
Urine ACR (mg/dL) (median [range])	45 [37–62]	51 [30–61]	
Renal Creatinine (mg/dL) (median [range])	138 [99.8–158.1]	121.5 [99.1–181.9]	
Estimated GFR (mL/min/1.73 m^2) (median [range])			
Medications (patient n-number)	4	3	—
Hydroxychloroquine	0	3	
Azathioprine	6	3	
Mycophenolate mofetil	5	5	
Prednisolone	0	0	
Methotrexate (oral)	1	0	
Rituximab	1	0	
Cyclophosphamide			

Table 3. Serum treatments for changes in ciGEnC IL-1β and IL-6 secretion. Age, gender, ethnicity, renal BILAG scores and medications for JSLE patients; age, gender and ethnicity for paediatric healthy controls (N = 6/group).

and subsequent Histopaque (Sigma-Aldrich) centrifugation of white blood cells. Neutrophils were re-suspended in RPMI (+10% FBS) at a concentration of 3×10^6/ml. ciGEnCs were then washed in PBS and following PBS removal 1 ml of neutrophil suspension was added onto each well. Cells were then incubated for 90 min at 37 °C. Following 90 min incubation, conditioned media containing non-adherent neutrophils were collected and neutrophils were counted using a cell counter. The cells remaining on the plate were washed in PBS and images were taken at 20x magnification. 5 random images per well were collected and the number of neutrophils adherent to the ciGEnC monolayer was counted using ImageJ software.

Immunofluorescence (IF). Transcription factor activation and nuclear translocation was assessed using IF. Following 30 min incubation, cells were fixed in 4% formaldehyde for 30 mins and permeabilised using 0.4% Triton-X-100 for 10 mins. Non-specific binding was blocked using PBS-1% BSA for 1 h (room temperature). Antibodies against human NF-κB, STAT-1 and STAT-2 were added overnight, at 4 °C. ciGEnCs were washed three times with PBS and incubated with fluorescence-conjugated secondary antibodies and 1 μg/ml of DAPI (4′,6-diamidino-2-phenylindole), for 2 h at room temperature. Cells were visualised immediately, using the EVOS FLoid Cell Imaging Station (ThermoFisher Scientific).

Image analysis. Image analysis to determine nuclear translocation of the signal in response to cytokine or LPS treatment was performed using Fiji (ImageJ) and particularly the Coloc2 algorithm[68]. For every group of treatments, the Pearson's r-values for every single cell, representing the degree of nuclear colocalisation between DAPI and A488 or A568, were obtained.

Statistical analysis. Statistical analysis of all data was performed using GraphPad Prism 4.0 software. Data are expressed as median values[range]. Multiple comparisons were made using Kruskal-Wallis or Friedman non-parametric test with Dunn's post-hoc test for three or more treatment groups and Mann-Whitney non-parametric test for two treatment groups. Statistical significance was set a priori at a p value < 0.05. For the IF assay, the r-values from each treatment group were used to perform non-parametric Kruskal-Wallis statistical test (with Dunn's post-hoc test) for three or more treatment groups, or Mann-Whitney tests for two treatment groups. For the nuclear colocalisation to be statistically significant, the r-values should range from 0.7 to 1 (0.7 indicated by dashed line in graphs).

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Author Contributions

Conceptualisation, P.D., R.D.W., M.W.B. P.D. designed all figures and drawings and performed all experiments for initial manuscript submission with support from R.D.W., A.M., M.P. and M.W.B. R.D.W. designed and performed the experiments for IL-6 and IL-1β detection in serum samples/ciGEnC conditioned media and investigation of MCP-1 and M-CSF levels in ciGEnC conditioned media after combined TNF-α/IL-1β treatments. R.D.W. and K.L.B. designed and performed the neutrophil adhesion assay. S.C.S. provided the ciGEnC cell line. P.D. wrote and edited the original draft. P.D., M.P., A.M., S.C.S., R.D.W. and M.W.B. all reviewed the original draft. P.D. edited the revised draft. R.D.W. and A.M. reviewed the revised draft. Funding was secured for this study by M.W.B. M.W.B. is the Chief Investigator of the UK JSLE Cohort Study and Repository, of which this study is part.

Additional Information

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Supplementary Figure and Table Legends

Supplementary Table 1. Synopsis of proteins produced by cytokine- and LPS-mediated stimulation of ciGEnCs.

Supplementary Table 2. List of antibodies.

Supplementary Table 3. List of primers.

Supplementary Figure 1. ciGEnC viability after 24 hours and 4 days of continuous TNF-α, IL-1β, IL-13, IFN-γ and LPS exposure. a. Viable (Anx V-/PI-), early apoptotic (Anx V+/PI-) and late apoptotic/necrotic (Anx V+/PI+) ciGEnCs after 24 hours of exposure to cytokines and LPS. N=5/group. **b.** Representative flow cytometric diagram.

Supplementary Figure 2. Full-length images of western blot gels for β-actin and Iκ-Bα expression after 30 minutes of cytokine and LPS stimulation. a. β-actin. **b.** Iκ-Bα. Unstimulated PMBCs were used as WB antibody controls.

Supplementary Figure 3. E-/P-selectin surface expression following 4- and 24-hour stimulation with TNF-α and IL-13. a. 4-hour E-/P-selectin surface expression following IL-13 and TNF-α treatments. **b.** 24-hour E-/P-selectin surface expression following IL-13 and TNF-α treatments. Data presented as median [range] are analysed using Kruskal-Wallis test with Dunn's post-hoc test, p=NS.

Supplementary Figure 4. Concentration response for a. TNF-α, **b.** IL-1β, **c.** IL-13 and **d.** IFN-γ.

Supplementary Table 1

FC Antibody (anti-human)	**Clone**	**Working Concentration**	**Catalogue Number, Company**
VCAM-1 - FITC (Mouse IgG1, kappa)	STA	4 µg/ml	11-1069, eBioscience/Affymetrix
ICAM-1 - APC (Mouse IgG1, kappa)	HA58	1 µg/ml	17-0549, eBioscience/Affymetrix
PD-L1 - PE (Mouse IgG1, kappa)	MIH1	2 µg/ml	12-5983-42, Invitrogen/Thermo Fisher Scientific
E-/P-selectin – PE (Mouse IgG1, kappa)	BBIG-E6 (13D5)	4 µg/ml	FAB6169P, R&D Systems/Bio-Techne
Isotype Control Antibody	**Clone**	**Catalogue Number, Company**	
Mouse G1 kappa- FITC	P3.6.2.8.1	17-4714, eBioscience/Affymetrix	
Mouse IgG1 kappa - APC	P3.6.2.8.1	17-4714, eBioscience/Affymetrix	
Mouse IgG1 kappa - PE	P3.6.2.8.1	14-4714-82, Invitrogen/Thermo Fisher Scientific	
IF Antibody (anti-human)	**Clone**	**Working Concentration**	**Catalogue Number, Company**
Rabbit RelA/NF-κB p65	Polyclonal	400 ng/ml	NB100-2176, Novus Biologicals/Bio-Techne
Mouse STAT-1	655210	780 ng/ml	MAB14901, R&D Systems/Bio-Techne
Rabbit STAT-2	Polyclonal	1.25 µg/ml	44-362G, Thermo Fisher Scientific
Secondary antibody	**Clone**	**Working Concentration**	**Catalogue Number, Company**
Goat anti-mouse - A568	Polyclonal	4 µg/ml	A-11004, Thermo Fisher Scientific
Goat anti-rabbit - A488	Polyclonal	4 µg/ml	A-11008, Thermo Fisher Scientific
WB Antibody (anti-human)	**Host species**	**Working Concentration**	**Catalogue Number, Company**
Rabbit IκBα (monoclonal)	rabbit	100 ng/ml	4812, Cell Signaling Technology
Mouse β-actin	mouse	333.3 ng/ml	ab8226, Abcam
Secondary Antibody	**Host species**	**Working Concentration**	**Catalogue Number, Company**
Goat anti-mouse IgG-HRP	Goat	20 ng/ml	HAF007, R&D Systems
Goat anti-rabbit IgG-HRP	Goat	20 ng/ml	HAF008, R&D Systems

Supplementary Table 2

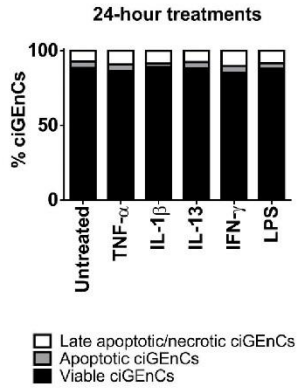
Gene	Forward Primer Sequence (5'- 3')	Reverse Primer Sequence (5'- 3')
ACTB	CATTGCGGTGGACGATGGA	AGATCAAGATCATTGCTCCTCCTG
TBP	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
TUBB	GGACCGCATCTCTGTGTACT	CTGCCCCAGACTGACCAAATA
IL-6	AGAGGCACTGGCAGAAAACA	TCACCAGGCAAGTCTCCTCA
IL-8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA
IL-10	GCGCTGTCATCGATTTCTTCC	GCCACCCTGATGTCTCAGTT
M-CSF	CCCCAGTCCTCTCTTAAAAGGC	GAGAGGACCCAGGCAAACTT
GM-CSF	GAGACACTGCTGCTGAGATGAA	AGGAAGTTTCCGGGGTTGGAG
MCP-1	CTCGCTCAGCCAGATGCAAT	TCTCCTTGGCCACAATGGTC
VCAM-1	GGTGGGACACAAATAAGGGT	GCTTGAGAAGCTGCAAAC
MIP-1α	CATTCCGTCACCTGCTCAGA	AGCAGCAAGTGATGCAGAGA
IP-10	AGCCCCACGTTTTCTGAGAC	GAGAGAGGTACTCCTTGAATGCC
TNF-α	CTTCTGCCTGCTGCACTTTG	GGGTTTGCTACAACATGGGC
IL-1β	CAACAGGCTGCTCTGGGATT	CCTGGAAGGAGCACTTCATCT
NF-κB	ACCCGGCTTCAGAATGGCA	AGGTATGGGCCATCTGCTGTT

Supplementary Table 3

Pro-inflammatory stimulus	Proteins produced by activated ciGEnCs
• **TNF-α**	M-CSF, GM-CSF, MIP-1α, MCP-1, Surface VCAM-1, ICAM-1
• **IL-1β**	IL-6, M-CSF, GM-CSF, MIP-1α, TNF-α, MCP-1, ICAM-1, ICOS-L
• **IL-13**	Soluble and cell surface VCAM-1
• **IFN-γ**	IL-10, IP-10, TNF-α, PD-L1
• **LPS**	IL-6, IL-10, IP-10, MIP-1α, IFN-γ, MCP-1, ICAM-1

Supplementary Figure 1

a.



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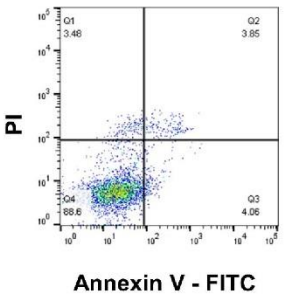
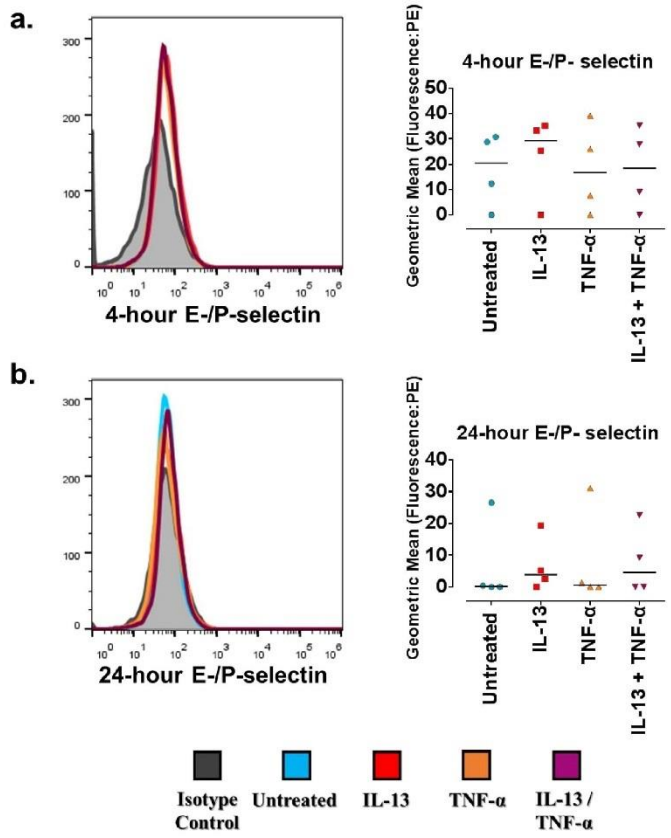
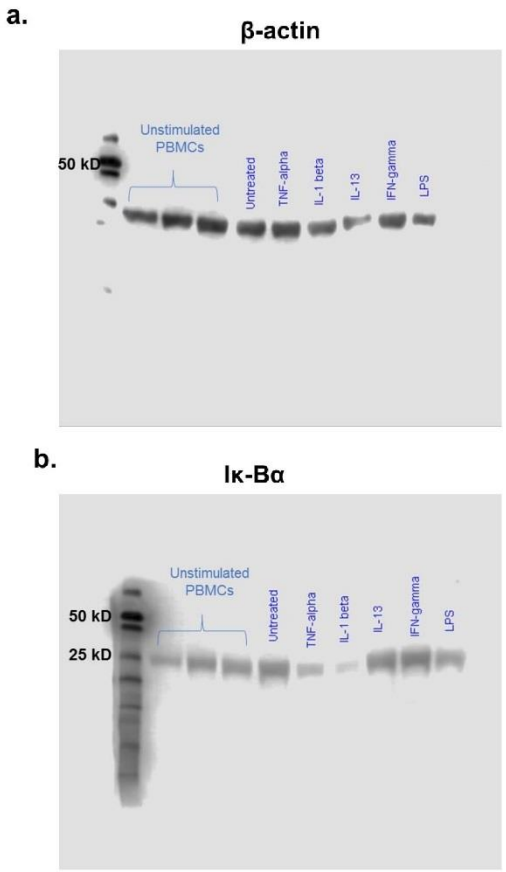


Supplementary Figure 2

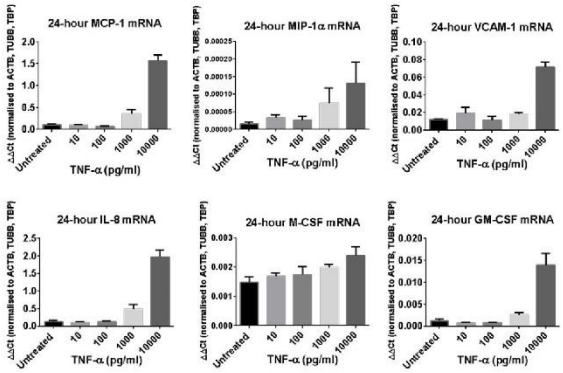


Supplementary Figure 3



Supplementary Figure 4

a.



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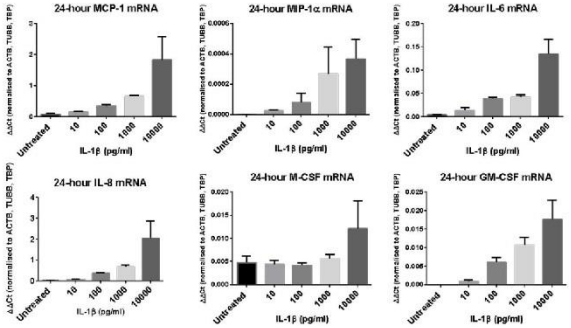

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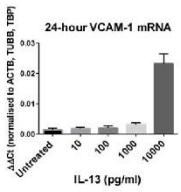

d.